# CHEMICAL, IMMUNOCHEMICAL, AND STRUCTURAL STUDIES OF CROSS-REACTIVE CELL WALL ANTIGENS OF *Streptococcus mutans*

By

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To my wife, Mary

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This dissertation is concerned with the isolation, purification, and immunochemical characterization of two key polysaccharide antigens of *Streptococcus mutans*, the principal etiologic agent of dental caries. These cell-wall antigens were isolated from two strongly cross-reacting serotypes (*a* and *d*) which have been confused by investigators. The information gathered here may aid in future reclassification or in the development of more accurate identification procedures for these oral pathogens. Also, the development of molecular vaccines composed of important somatic antigens depends on knowledge of antigen composition and structure.

The principal cross-reacting wall antigens of *S mutans* strains AHT (serotype *a*) and B13 (serotype *d*) were extracted from purified cell walls by hot formamide. The antigens were purified by affinity chromatography using the galactose-binding lectin from *Ricinus communis*. The purified antigens appeared to be immunologically identical to crude antigen extracts of whole cells or cell walls. The antigens were

composed primarily of galactose and glucose in molar ratios of 3.4:1 for AHT, and 2.1:1 for B13. Rhamnose, which is the major constituent of the crude formamide extracts, was present in only trace amounts in the lectin purified preparations. Hapten inhibition using absorbed antisera specific for the unique antigenic determinants ($a$ or $d$) and the cross-reacting determinants ($a$-$d$) showed that the $a$ specificity depended on an α-linked galactose residue, possibly linked to glucose. In the case of the $d$ determinant both glucose and galactose allowed significant inhibition, however, glucose gave slightly greater inhibition.

The cross-reacting $a$-$d$ determinant appears to be galactose, possibly linked 1→6 to glucose. Methylation analysis of the antigens using combined gas-liquid chromatography-mass spectrometry showed that both antigens possess identical branch point residues as well as three other identical residues including terminal galactose moieties which may be responsible for the cross reaction. The AHT antigen contains a 1→3 linked galactose residue and B13 contains a 1→3 linked glucose residue which may determine the $a$ and $d$ specificities respectively. Both antigens contain galactofuranose residues which may contribute to the observed acid-lability of these antigens.

The data presented in this dissertation provide some clues concerning the possible molecular structures of the $a$ and $d$ antigens. Due to the complexity of each polymer, it is not yet possible to draw complete structures. There does appear, however, to be a structure common to each antigen which will be proposed in this dissertation. Finally, our immunochemical studies of the $a$, $d$, and $a$-$d$ haptenic sites (by precipitin inhibition analysis) have failed to confirm published data or have revealed contrary information.

# INTRODUCTION

*Streptococcus mutans*, a Gram-positive coccus belonging to the viridans group, is considered to be the principal etiological agent in dental caries. Its ability to colonize tooth surfaces by the production of a sticky dextran-like capsule and the ability to produce acid from fermentable carbohydrates account for the cariogenicity of this organism. Dental caries is the most prevalent disease of civilized populations and poses serious health and financial problems. Gibbons (29) states "It has been estimated that well over two billion dollars are spent each year in the United States for the treatment of this disease, and thus tooth decay ranks as one of the most expensive bacterial infections of mankind." Accordingly, a great deal of work has been done on the classification of *S. mutans* and toward the development of vaccines for these organisms. One of the foremost ways of classifying *S. mutans* strains is serologically. This, and the potential development of vaccines, necessitates a good understanding of the antigens associated with the various strains. This dissertation will present chemical, immunochemical, and structural data on purified antigens of two cross-reacting strains of *S. mutans* of the serotypes *a* and *d*.

Classification of *S. mutans* strains. The various cariogenic and most other oral streptococci are classified as belonging to the viridans group, which give greening on blood agar and are not classifiable by the Lancefield grouping scheme.

Early investigations in 1960 by Fitzgerald and Keyes (27) and Fitzgerald et al. (26) showed that cariogenic isolates from rats and hamsters could be distinguished by their ability to ferment sorbitol, but antigenic extracts failed to react with antisera used for the identification of Lancefield groups A-H and K-S (37). Zinner et al. (64) isolated various cariogenic strains from human sources and also found them to be non-reactive with Lancefield grouping sera.

*S. mutans* strains form a relatively homogeneous group when classified according to biochemical reactions. Most strains ferment sorbitol and mannitol, as well as melibiose, salicin, and inulin, and produce large amounts of non-dialyzable glucan from sucrose. They also hydrolyze esculin, grow in 4% NaCl broth, and on 10% bile agar. Using cluster analysis, Colman and Williams (15) showed that among 216 non-hemolytic streptococci the dectran-producing organisms formed a separate cluster comprised of mostly *S. mutans*. Guggenheim (30) and Edwardsson (25) also showed *S. mutans* to form a homogeneous group by biochemical tests. Drucker and Melville (22, 23), and Carlsson (13) showed by numerical taxonomy that *S. mutans* was a distinct group and could be distinguished from other common oral streptococci: *S. sanguis*, *S. mitis*, and *S. salivarius*.

When examined by serological and genetic methods, *S. mutans* as a group appears more heterogeneous. Jablon and Zinner (36, 63) examined eight strains of cariogenic streptococci using immunofluorescence and were able to place them in four groups, I-IV. However, there were cross-reactions with other streptococci making the technique less useful than subsequently developed techniques for the identification of *S. mutans*.

Perhaps the most useful method to date of classifying strains of *S. mutans* was the serological scheme developed by Bratthall (9, 10, 11) utilizing diffusion in gel and comparative immunoelectrophoretic patterns of extracts perpared according to Lancefield (37). Although cross-reactions existed, as were seen by other workers, the use of comparative immunoelectrophoresis allowed Bratthall to separate 70 strains of *S. mutans* into five serotypes: *a* through *e*. The serotype *e* organisms cross-reacted using anti-Lancefield group E serum. The serotypes *a* through *d* did not react with any of the Lancefield grouping sera. In a subsequent study, Bratthall (12) used antisera conjugated with fluorescein isothiocyanate (FITC) and found that unabsorbed, conjugated antisera prepared against reference strains of the five serotypes showed cross-reactions with members of the other serological groups of *S. mutans* as well as members of other streptococcal species. Most notably, anti-AHT or anti-3720 (*a*) serum showed a strong cross-reaction (4+) with B13 (*d*). Anti-B13 serum showed a moderate cross-reaction with the same serotype *a* organisms (2+). Anti-JC2 serum (*c*) showed a strong cross-reaction (3+ to 4+) with LM7 (*e*), while anti-SS9 serum, a Lancefield group E serum, reacted weakly (1+) with JC2 (*c*) and B13 (*d*), and strongly (3+ to 4+) with LM7. Through various whole cell absorption procedures Bratthall was able to eliminate cross-reactions with non-*S. mutans* species and produce specific staining sera for all serotypes except *c*, which still reacted with LM7 (*e*). The *S. mutans* serotype *e* cells could be identified by absorbing antiserum against Lancefield group E (strain SS9) with *S. mutans* LM7 (*e*). Then a *S. mutans* strain of serotype *e* will react with unabsorbed anti-SS9 serum, with anti-JC2 (*c*) serum, but not with the absorbed anti-SS9 serum. On the other hand, Lancefield group E strains react with both the absorbed and unabsorbed anti-SS9

serum, but not with anti-JC2 (*c*) serum. The serotype *c* organisms do not react appreciably with either anti-group E serum.

de Stoppelaar (20) was able to divide various cariogenic streptococci into three groups by precipitin analysis. His groups MI, MII, and MO correspond to Bratthall's serotypes *c*, *a*, and *e* respectively.

Further work by Perch et al. (48) demonstrated two more serotypes, *f* and *g*, using absorbed fluorescine-labelled antisera.

In addition to the serological grouping schemes, four genetic groups based on DNA-DNA hybridization and % guanine-cytosine content were shown by Coykendall (16, 17, 19) to parallel four of Bratthall's five serotypes. Coykendall (18) proposed the adoption of subspecies names for his genetic groups I through IV: *S. mutans var. mutans, var. rattus, var. sobrinus,* and *var. cricetus* respectively. Shklair and Keene (54) separated *S. mutans* into five biotypes, *a-e,* which correspond directly with Bratthall's serotypes. Biotypes were determined by fermentation patterns using mannitol, mannitol + bacitracin, sorbitol, raffinose, melibiose, and production of ammonia from arginine.

Table 1 summarizes the relationships among the strains of *S. mutans,* determined by various workers, using serological methods, DNA-DNA reassociation (genetic groups), guanine-cytosine content (%GC), and biochemical reactions (biotypes).

Immunochemical nature of the serotype antigens. In 1973 Van de Rijn and Bleiweis (59) isolated a glycerol teichoic acid antigen from *S. mutans* AHT (*a*) by cold 5% trichloroacetic acid (TCA) extraction. The material was fractionated by agarose gel chromatography and the antigen fraction contained glycerol and phosphorus in a 1:1 molar ratio. The fraction also contained glucose, galactose, alanine, and fatty acids.

Table 1. SURVEY OF THE VARIOUS CLASSIFICATION SCHEMES FOR *Streptococcus mutans*

| Serotypes (10)[a] | Serotypes (48) | Serotypes (20) | Serotypes (36) | Genetic groups (17) | % GC | Subspecies (18) | Biotypes (54) |
|---|---|---|---|---|---|---|---|
| *a* | | MII | I | IV | 42-44 | *var. circetus* | *a* |
| *b* | | | II | II | 41-43 | *var. rattus* | *b* |
| *c* | | MI | | I | 36-38 | *var. mutans* | *c* |
| *d* | | MII | | III | 44-46 | *var. sobrinus* | *d* |
| *e* | | MO | | | | | *e* |
| | *f* | | | | | | |
| | *g* | | | | | | |

[a]See *Literature Cited*

Partial alkaline hydrolysis yielded a glyceride containing a disaccharide of glucose and galactose attached to the 2-hydroxyl group of glycerol. Precipitin inhibition analysis showed that β-galactosides were the greatest inhibitors (> 75%) while glucose and other glucosides inhibited to a lesser extent (< 30%). Immunodifussion and immunoelectrophoresis showed this antigen to be present in all six serotype *a* organisms tested and absent in serotypes *b*, *c*, and *d*.

A short while later, in 1973, Mukasa and Slade (46) isolated a polysaccharide antigen from lyophilized whole cells of a hamster strain of *S. mutans*, HS6 (*a*), using boiling water. The ethanol precipitate of the extract was purified by chromatography on diethylaminoethyl (DEAE)-Sephadex A-25, Sephadex G-200, and Carboxymethyl (CM)-Sephadex C-25. The antigen was primarily composed of galactose (54%), glucose (10.4%), glucosamine (8.6%), and galactosamine (5.4%), with small amounts of lipid, phosphorus, and 5% amino acids. The antigen reacted not only with anti-HS6 serum but also with anti-B13 (*d*) serum. The cross-reactivity could be eliminated by absorbing anti-HS6 serum with B13 whole cells. Cross-reactive ("*a*-*d*") serum could be obtained by releasing the antibody that bound to B13 cells during absorption. In subsequent papers, Linzer et al. (39, 41) showed that the purified antigen possessed two sites on a single molecule: the "*a*" site, unique to the *a* serotype, and an "*a*-*d*" site, which was responsible for the cross-reaction with *d* serotypes. Precipitin inhibition studies with abosrbed anti-HS6 serum showed the "*a*" site to be dependent on α-1→4 and β-1→6 glucosides, however, free galactose or galactosides other than lactose were not tested as potential inhibitors. The latter disaccharide gave less than 10% inhibition. Maltose (glu α-1→4 glu) and gentiobiose (glu β-1→6 glu) each

gave approximately 50% inhibition, while α and β-methylglucopyranosides gave less than 10%. Inhibition studies with "*a-d*" serum showed 32% inhibition with galactose, 36% with galactosamine, and 24% with melibiose. No other inhibitors were tried. Since this dissertation deals with the relationship of the antigens of serotypes *a* and *d* in great detail, including structural analyses of the purified antigens, the reports outlined above (41, 46) will be further discussed below (see *Discussion*).

In 1973, Mukasa and Slade (45) isolated two antigens from whole cells of a serotpye *b* organism FA1, using cold 10% TCA. One (antigen 1) was a polysaccharide and the other (antigen 2) was a mucoprotein. Both antigens possessed the same immunological specificity. Both antigens contained about 30% galactose and 3% galactosamine, while rhamnose was the major component (47%) of the carbohydrate antigen (antigen 1) and present only in traces in antigen 2. Antigen 2 contained about 40% protein while antigen 1 contained 5% protein. When studied by immunoelectrophoresis, the two antigens migrated as individual bands, with antigen 2 migrating the farthest toward the anode. Galactose and galactosamine were the best precipitin inhibitors (78%) of antigen 2 reactivity. Antigen 1 was not studied for immunochemical specificity.

In 1974, Vaught and Bleiweis (60) isolated a glycerol teichoic acid antigen from purified cell walls of *S. mutans* BHT (*b*) using cold 10% TCA. The extract was fractionated by gel filtration and ion exchange chromatography. Two fractions were obtained, each yielding a single separate band on immunoelectrophoresis which migrated in the same manner as the bands obtained from mild acid extracts of whole cells of serotype (*b*) organisms. The faster migrating band, fraction C, was similar in mobility to Slade's antigen 2 except fraction C contained primarily

galactose with glycerol and phosphorus in a molar ratio of 1.0: 1.4. Only small amounts of rhamnose and glucose were measured. This fraction resembled a glycerol teichoic acid. Fraction B was very similar to Slade's antigen 1, both in mobility and in chemical content.

In 1974, Wetherell and Bleiweis (61) isolated a polysaccharide antigen by hot formamide extraction from purified cell walls of *S. mutans* GS5 (*c*). The major antigen, purified by gel filtration and ion exchange chromatography, was composed mainly of rhamnose and glucose in a molar ratio of 1.7: 1.0. Precipitin inhibition studies revealed the major hapten to be an α-linked glucose moiety. In a subsequent study, Linzer et al. (40) isolated a nearly identical polysaccharide from hot 5% TCA extracts of whole cells of strain Ingbritt, another serotype *c* organism.

Linzer and Slade (42) isolated a polysaccharide antigen from whole cells of *S. mutans* strain B13 (*d*) using cold 5% TCA. After the antigen was purified by ion exchange and gel filtration chromatography, it was found to be composed mainly of galactose and glucose in a 2:1 molar ratio. The antigen was similar to the *a* antigen in that it was a single molecule possessing two reactive sites: one specific for the *d* serotype (*d* site) and one responsible for the cross-reaction with the *a* antigen (*a*-*d* site). Anti-B13 serum could be abosrbed with HS6 (*a*) cells to yield a specific *d* serum. Inhibition studies with anti-*d* serum (absorbed) showed the hapten to be galactose, possibly α-linked. The nature of the *a*-*d* site specificity in B13 was not investigated.

The serotype *e* antigen was purified after hot formamide extraction of purified cell walls of V-100 by Wetherell and Bleiweis (62). Two polysaccharide antigens were resolved by ion-exchange chromatography: one antigen (I) was specific for serotype *e*, and the other (II) cross-

reacted with serotype *c* antisera. Antigen I was composed mainly of rhamnose and glucose in a 2.3:1 molar ratio and possessed a β-glucosyl group as its antigenic determinant. Antigen II possessed identical chemical components and its homologous reactivity with anti-*e* serum also was inhibited primarily by β-glucosides. Although not proven by the authors, it was speculated antigen II may possess α-glucosyl haptenic moieties to account for cross-reactions with *c* serum. Hamada and Slade (33) isolated a similar antigen from strain MT703 (*e*) by the autoclave extraction method of Rantz and Randall (51). The major antigen, *e*I was composed mainly of rhamnose (56%) and glucose (37%) and possessed a β-glucoside as a haptenic determinant. The *e* - *c* cross-reactive antigen was not found.

Hamada et al. (32) isolated a polysaccharide antigen with serotype *f* specificity from whole cells of strains OMZ175 and MI557 using hot 5% TCA. The antigen from OMZ175 was composed of rhamnose (49%) and glucose (47%), and the antigen from MI557 also contained rhamnose and glucose, 60% and 39% respectively. The best inhibitor of precipitation was isomaltose, a diglucoside with an α1→6 linkage. The antigen was cross-reactive with the glucan ("mutan") produced by the same organism when grown in sucrose.

A polysaccharide antigen from a pH 7.3 buffer-boiled extract of *S. mutans* 6715 (*g*) was isolated by Iacono et al. (35) in 1975. At the time this strain was thought to belong to serotype *d*, but is now considered to be a serotype *g* organism (34, 48). The antigen, purified by ethanol precipitation and gel filtration, was composed primarily of galactose (60.6%) and glucose (10.3%) and amino acids (9.5%). The composition was very similar to the *a* antigen of Mukasa and Slade (46)

and indeed serotype *g* strains have been shown to cross-react with both serotype *a* and *d* strains. It is not known whether the molecular site responsible for the *a-g* and *d-g* cross-reaction is the same as that for the *a-d* cross-reaction. Precipitin inhibition tests showed that galactose was the most effective inhibitor, although glucose also gave significant inhibition. The immunodominant region is thought to involve the 1→6 linkage of galactose and glucose in either the α or β configuration.

The work presented in this dissertation began in 1973, prior to the publication of work on the polysaccharide antigens of serotypes *a* and *d*. Because of major discrepancies between the published data and our early findings, and more importantly because of a desire to gain information concerning the basic structural nature of these antigens, work was continued. The findings in this dissertation agree in part with some of the published data on the *a*, *d*, and *a-d* antigens, but they also refute, or at least open to further debate, previous findings heretofore unverified. In addition, the structural data presented below which have not previously been published, provide further insight into the chemical structure of these antigens and may provide important information to future investigators desiring to synthesize immunogens for anti-caries vaccines or to more fully understand the immunogenicity and antigenicity of surface polysaccharides of *S. mutans*.

## MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. mutans* strain AHT (*a*) was obtained from J. Jablon (University of Miami, Miami, Florida), and strain B13 (*d*) was obtained from D. Bratthall (University of Göteborg, Göteborg, Sweden).

Cultures were maintained as lyophilized stocks. Samples to be lyophilized were grown for 20 h at 37°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Michigan) supplemented with 0.4% glucose (THG), and adjusted to pH 6.5 with concentrated HCl before autoclaving. The pellet from 250 ml of broth was suspended in 2 ml of sterile 20% powdered milk solution (Pet, Inc., St. Louis, Missouri), and 0.2 ml samples were lyophilized in glass ampoules on a Virtis freeze drying apparatus (Virtis Research Equipment Co., Gardiner, New York). The ampoules were sealed under vacuum and stored at -20°C.

Cells for antigen preparation were grown in ten liter batches in THG. Growth was initiated by inoculating 10 ml of THG from lyophilized stocks. The entire culture was transferred at 24 h intervals into 250 ml, 1000 ml, and finally 8750 ml of THG giving a total volume of 10 l. Cells were harvested 24 h after the last transfer in a Delaval gyro-testor (Delaval Separator Co., Poughkeepsie, New York). Autolysins were inactivated by heating the cells at 60°C for 20 min.

Extraction of antigens for routine identification. Lancefield-type acid extracts were used for routine identification. Approximately 5 mg of whole cells or cell walls were suspended in 1 ml of saline and

adjusted to pH 2.5 with HCl. The suspensions were boiled for 10 min, cooled, centrifuged at 1070 x g for 20 min to remove cell debris and the supernate neutralized with 0.05 N NaOH.

Cell walls used for extraction were prepared by the method of Bleiweis et al. ( 7 ) which involves breakage with glass beads in a Braun tissue homogenizer (Bronwill Scientific, Rochester, New York), followed by treatments with ribonuclease, deoxyribonuclease, and trypsin.

Extraction of antigens for purification. Antigens for further purification were extracted by the hot formamide method of Fuller (28). Cell walls (800 mg) were suspended in 40 ml formamide and heated at 180°C for 30 min in a paraffin oil bath. The suspension was then washed by adding 2 vol (80 ml) of an acidic ethanol solution containing 19 parts absolute ethanol and 1 part 2N HCl (vol/vol), and centrifuging at 7700 x g for 20 min in glass centrifuge tubes. The supernate was decanted and saved and the pellets were washed twice more with 30 ml of a mixture of the above acid-ethanol solution and deionized water (2:1, vol/vol). Acetone (900 ml) was added to the combined supernates and the resulting solution was immediately poured into round bottom centrifuge bottles (250 ml capacity). One milliliter of 1M NaCl was added to each bottle and the bottles were stored at 4°C for at least 8 h. The bottles then were centrifuged at 530 x g for 20 min, the supernates were decanted and the pellets were allowed to dry. The pellets were dissolved in deionized water, centrifuged at 27,000 x g for 20 min, and the supernates were dialyzed and lyophilized.

Isolation of castor bean lectin (CBL). The castor bean lectin from *Ricinus communis* was isolated by the method of Nicolson and Blaustein (47) for use in affinity chromatography. Extracts of castor

beans are extremely toxic and allergenic and, accordingly, care must be taken in handling them and all glassware was detoxified using a dilute solution of sodium hypochlorite.

*R. communis* beans, 100 g (kindly provided by the McNair Seed Co., Plainview, Texas), were blended in a model 1120 Waring blender (Waring Products Div., Dynamics Corporation of America, New Hartford, Connecticut) with 1 l of 0.005 M sodium phosphate buffer pH 7.2 containing 0.2 M NaCl and $10^{-4}$ M $Mg^{+2}$, $Ca^{+2}$, and $Mn^{+2}$ as chlorides ($PBS^{++}$). The beans were blended into a smooth paste (ca. 30 min), then the extract was filtered through cheesecloth and centrifuged at 10,000 x g for 30 min at 4°C. The supernate was carefully decanted and saved, without including the floating lipid layer, and centrifuged again. The supernatant solution was adjusted to 60% saturation with ammonium sulfate and slowly stirred at 4°C overnight. After centrifugation at 10,000 x g for 20 min, the pellet was dissolved in $PBS^{++}$ and dialyzed against $PBS^{++}$ to remove the ammonium sulfate. The dialysate (ca. 50 ml) was then applied to a 4 x 40 cm column of Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey) and eluted with $PBS^{++}$ until the absorbance at 280 nm was less than 0.05. Since Sepharose 4B contains polysaccharides with galactose-terminal groups, it served as an affinity column to bind the lectin. The lectins ( a mixture of two, $RCA_{60}$ and $RCA_{120}$) then were eluted with 0.2 M D-galactose in $PBS^{++}$. The $A_{280}$ peak was pooled, dialyzed against $PBS^{++}$ and then deionized water, and finally lyophilized.

Preparation of affinity columns with castor bean lectins. An affinity chromatography gel was prepared using Affi-Gel 10 (Biorad Laboratories, Richmond, California), an agarose gel containing a 10-Å aliphatic spacer arm terminating with an active carboxy-N- hydroxy-

succinimide ester group. Approximately 350 mg of the lectin was dissolved in 50 ml of 0.1 M sodium phosphate buffer pH 7.0 containing $10^{-4}$ M metal ions as before, and 0.05 M D-galactose, to prevent binding of the lectin to the gel matrix. To each of two vials of gel (approximately 13 ml wet gel volume per vial), 25 ml of the lectin solution was added and the vials were gently agitated on a shaker for 24 h at 4°C. The coupled gel was packed into a 1.3 x 30 cm column and eluted with $PBS^{++}$ plus 0.2 M D-galactose until the absorbance at 260 nm reached baseline, indicating the N-hydroxy-succinimide released during coupling was removed. The column was washed with about 200 ml of $PBS^{++}$ minus galactose before loading with a sample.

Purification of antigens by affinity chromatography. Samples of crude formamide extract (50 mg) were loaded on the affinity column at 4°C and eluted with $PBS^{++}$. The flow rate was 12 ml/h and 2 ml fractions were collected. After 72 ml were collected to remove non-binding materials, the elution system was changed to $PBS^{++}$ plus 0.2 M D-galactose to remove bound polysaccharides. The column was eluted with at least 100 ml of the latter buffer before re-equilibrating with the starting buffer. [All buffers used with the affinity columns were pre-filtered through a 0.45 µm membrane filter to prevent clogging of the bed surface].

Fractions were monitored for carbohydrate by the phenol-sulfuric acid method as described below and for antigenic reactivity by capillary tube precipitation using the appropriate antiserum.

Gel filtration chromatography. Samples of crude formamide extract (50 mg) were loaded on a 2.5 x 80 cm column of Bio-Gel P-150 (Biorad) 100-200 mesh and eluted with 0.85% NaCl at a flow rate of 10 ml/h. Fractions (2 ml) were collected and analyzed for absorbance at 220 nm, rhamnose as described below, and antigenic reactivity using capillary precipitin tubes.

Samples of lectin-purified antigen were filtered as above except on 2.5 x 40 cm columns. Fractions (2 ml) were collected and assayed for sugars by the phenol-sulfuric assay and for antigenic reactivity by precipitation in capillary tubes.

Quantitative chemical assays. Phosphorus in cell walls was measured by the method of Chen et al. (14), and for the purified antigens by the method of Lowry et al. (43). Both methods depend on the formation of a blue phosphomolybdate complex, but differ in the ashing procedure and in sensitivity.

Determination of glucose and galactose in cell walls was performed using the Glucostat and Galactostat reagents (Worthington Biochemical Corp., Freehold, New Jersey). Samples were hydrolyzed in 3 ml of 2 N HCl for 4 h at 100°C. Hydrolyzed samples were quantitatively transferred to a 10 ml volumetric flask and neutralized to a phenolpthalein endpoint with 5 N NaOH. The volume at this point was 7.6-7.8 ml. The sample was then brought to 10.0 ml with 0.27 M sodium phosphate buffer, pH 7.0.

Rhamnose in cell walls and column fractions was measured by the method of Dische and Shettles (21).

Glycerol was determined utilizing the Glycerol Stat-Pack (Calbiochem, Atlanta, Georgia) according to the manufacturer's instructions. Samples of cell walls (10 mg) were hydrolyzed for 3 h at 105°C in 1 ml of 2 N HCl. After hydrolysis, the acid was removed by lyophilization and three 1.5 ml distilled water washes, each followed by lyophilization. Free glycerol was released by treating the samples with 2 mg alkaline phosphatase (Worthington) for 24 h at 37°C in 3 ml of 0.08 M ammonium carbonate buffer pH 9.3.

The phenol-sulfuric acid assay was performed according to Dubois et al. (24) using glucose as a standard. The tubes were read at 485 nm.

Protein was determined by the method of Lowry et al. (44). Details of the assay are given below.

Amino acids and amino sugars were determined using a JEOL model JLC-6AH automated amino acid analyzer (JEOL, Inc., Cranford, New Jersey) according to the method of Spackman et al. (58). Peaks were quantitated using an Autolab System AA integrator (Spectra-Physics, Mountain View, California). Samples (10 mg) were hydrolyzed in sealed ampoules with either 5 ml of 4 N HCl for 11 h at 105°C or with 5 ml of 6 N HCl for 18 h at the same temperature. The second method was necessary for the analysis of cell walls of serotypes *a* and *d* in order to fully hydrolyze an acid resistant component presumed to be a lysyl-threonine dipeptide (8). After hydrolysis, acid was removed using a rotary evaporator (Buchler Instruments, Fort Lee, New Jersey) and the samples were washed three times with deionized water followed by evaporation. Hydrolyzed samples were quantitatively transferred to a 25 ml volumetric flask and diluted to volume using 0.01 N HCl.

Gas liquid chromatography of sugars. Sugars in the crude formamide extracts and purified antigens were measured by gas liquid chromatography. Samples (1-5 mg) were hydrolyzed in sealed ampoules with 1 N $H_2SO_4$ for 8 h at 100°C. After hydrolysis, the samples were cooled, opened, and 1.0 ml of a mannitol solution of a known concentration (usually 5.5 µmole/ml) was added as an internal standard. The hydrolysates were neutralized with $BaCO_3$, and the precipitate of insoluble $BaSO_4$, as well as any excess $BaCO_3$, was removed by centrifugation at 1000 x g for 20 min. Supernates were centrifuged again as above and then lyophilized.

Hydrolysates were converted to their trimethylsilyl derivatives using TRI-SIL (Pierce Chemical Co., Rockford, Illinois) according to the instructions in the Pierce handbook of silylation (49). The derivatives were separated on 6 ft x 4 mm glass columns packed with SE-30 Ultraphase, 3%, on Chromosorb W (HP) 80/100 mesh (Pierce) on a Packard model 803 gas-liquid chromatograph (Packard Instrument Co., Downers Grove, Illinois). The operating temperature was 165°C (isothermal), the carrier gas (nitrogen) flow rate was 30 cc/min, and a hydrogen flame ionization detector was used. Peaks were quantitated using an Autolab Minigrator electronic digital integrator (Spectra-Physics, Santa Clara, California). Calibration curves were constructed by injecting samples containing varying amounts of the standard sugars deriviatized as above and a constant amount of the internal standard, mannitol. The ratio of area mannitol/area standard was plotted *vs.* µmoles mannitol/µmoles standard.

Methylation analysis of polysaccharide antigens. Purified antigens were methylated using the method of Hakomori (31) according to Bjorndal et al. (4) and Lindberg (38). The procedure involves treating the polysaccharides with methylsulfinyl sodium in dimethyl sulfoxide (DMSO), followed by methylation with methy iodide. The methylated product is hydrolyzed, reduced, acetylated and analyzed by gas liquid chromatography and mass spectrometry. All solvents were re-distilled and stored in glass-stoppered bottles and care was taken to prevent contamination with plastics.

The methysulfinyl carbanion was prepared according to Sanford and Conrad (53) and stored at -20°C under mineral oil.

Samples of antigen (1.5 mg) were placed in small serum vials and capped with rubber septa. The vials were flushed with dry nitrogen through an 18-gauge hypodermic needle and the samples dissolved in 1.0 ml of DMSO

which had been dried over 3 Å molecular sieves (Davison Chemical, Baltimore, Maryland). Methylsulfinyl sodium, 1.0 ml, was introduced into the vials with a glass syringe and the mixture was treated for 1 h with ultrasound at room temperature. The reaction was allowed to proceed for an additional 6 h with stirring, and then methyl iodide (1.0 ml) was added dropwise with cooling in an ice bath. The solution was agitated in an ultrasonic bath for 30 min, diluted with 10 ml of deionized water, dialyzed overnight against 10 l of deionized water, and evaporated to dryness on a rotary evaporator at 40°C. The above procedure was repeated using the product of these reactions to ensure complete methylation.

The methylated polysaccharides were hydrolyzed in two steps. The residue was dissolved in 3 ml of 90% formic acid and treated at 100°C for 2 h. The samples were evaporated to dryness at 40°C and then dissolved in 1 ml of 1 N $H_2SO_4$ and hydrolyzed for 8 h at 100°C. The hydrolysate was neutralized with barium carbonate, clarified by centrifugation and evaporated to dryness at 40°C.

The residues were dissolved in 5 ml of deionized water containing 15 mg sodium borohydride and allowed to stand at room temperature for 2 h. To the mixture was added 1 ml (wet volume) of AG 50W-X8, $H^+$ form, (Biorad). The mixture was agitated for 5 min, filtered, and evaporated to dryness at 40°C. The residual boric acid was removed by codistillation with methanol (5 x 5 ml) on the rotary evaporator. Finally, the mixture of reduced, methylated sugars was acetylated with acetic anhydride-pyridine, 1:1, for 15 min at 100°C. The reagents were removed by distillation with toluene (5 x 5 ml) on the rotary evaporator at 40°C. The residue was dissolved in 0.1-0.2 ml acetone for analysis.

Analysis of methylated sugar derivatives. The partially-methylated alditol acetate derivatives were separated by gas liquid chromatography on 6 ft x 2 mm silanized glass columns packed with 3% OV-225 or 3% ECNSS-M on Gas-Chrom Q,100-120 mesh (Applied Science Laboratory, Inc., State College, Pennsylvania). Temperature for determination of relative retention times was 170°C, and 155°C for measurement of peak area. The nitrogen carrier flow rate was 20 cc/min and a hydrogen flame ionization detector was used. Analysis was carried out on a Packard model 803 gas-liquid chromatograph (Packard Instrument Co.). Retention times relative to 1,5-di-*O*-acetyl-2, 3,4,6-tetra-*O*-methyl-D-glucitol were determined by interpolation between known standards prepared from a mixture of 2,3,4,6-tetra-*O*-methyl-D-glucose (Sigma Chemical Co., St. Louis, Missouri) and methylated, hydrolyzed, lactose (Sigma) and oyster glycogen (Nutritional Biochemical Corp., Cleveland, Ohio). This mixture provides standards of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol.

Confirmation of peak identity was obtained by combined GLC-mass spectrometry on a Pye model 104 gas chromatograph (Pye Unicam Ltd., Cambridge, England) interfaced with an Associated Electrical Industries model MS 30 double-focusing mass spectrometer with a DS-30 data system (AEI, Scientific Apparatus Ltd., Manchester, England). Peaks were detected by following total ion current. Gas chromatographic conditions were as described above, using OV-225 as the stationary phase and a column temperature of 170 or 200°C. Helium was used as the carrier.

Preparation of antisera. Cells of each strain (AHT, B13) were grown for 20 h in 250 ml of THG in plastic centrifuge bottles. Cells were pelleted by centrifugation at 10,000 x g for 15 min and washed twice with sterile saline. The cells were resuspended in 100 ml of saline containing 0.6% formalin and incubated for 24 h at room temperature. The cells were washed twice with sterile saline and diluted in saline to a reading of 360 using a #66 filter on a Klett-Summerson model 800-3 colorimeter (Klett Manufacturing Co., Inc., New York, New York). After noting the volume, the cells were pelleted as above and resuspended in one-tenth of the original volume in sterile saline. The suspensions were stored at -20°C and used as immunogens for antiserum production.

Male New Zealand white rabbits (3 kg) were injected intravenously on Monday, Wednesday, and Friday according to the following regimen: during the first week 0.1 ml was injected, the second week 0.2 ml, and the third week 0.3 ml. Rabbits were given a prophylactic intramuscular injection of 1 ml (50 mg/ml) of Liquamycin (oxytetracycline-HCl, Pfizer, Inc., New York, New York) after each immunization. On Monday of the fourth week animals were test-bled from the ear artery and the serum was tested for ability to precipitate with the homologous, cell-wall Lancefield extract in capillary tubes, and for cross-reactivity by Ouchterlony gel-diffusion analysis. Animals with suitable titers were anesthetized with pentothal sodium and exsanguinated by heart puncture. Sera were stored in small aliquots at -20°C with 0.01% sodium azide.

Gel diffusion and immunoelectrophoresis. For both gel diffusion and immunoelectrophoresis (IE), gels containing 0.75% agarose (Sigma) in 0.05 M sodium barbital/HCl pH 8.2 were used. Slides for IE (7.6 x 3.8 cm) were cleaned in chromic acid, dipped in 0.2% agarose in deionized water

and allowed to dry. The buffered, molten 0.75% agarose solution (3 ml) was pipetted onto each slide and allowed to solidify. Wells and troughs were cut in the slides using a Shandon gel cutter (Shandon Scientific Co. Ltd., London, England). Electrophoresis was performed at 4°C at 10 ma per slide for 120 min using a VOKAM SAE 2761 power supply (Shandon) and employing the above barbital buffer. After electrophoresis of the antigen, the troughs were cleared of agarose, filled with the appropriate antiserum, and the plates were incubated at 4°C in a moist chamber.

For gel diffusion analysis, plastic petri dishes, 50 x 12 mm, (Falcon No. 1006, Falcon Plastics, Oxnard, California) were filled with 3 ml of the same buffered agarose solution and the appropriate number of wells (50 µl capacity) were cut. After addition of antigen and antiserum preparations to the wells, plates were incubated at 20°C until development of precipitin bands.

Absorption of antisera. Whole antisera were absorbed with an equal volume of heat-killed, washed, THG-grown cells of the heterologous strain for 4 h at 4°C with stirring. The cells were pelleted by centrifugation at 8000 x g for 20 min. The supernates contained antibodies specific for the $a$ (AHT) or the $d$ (B13) determinants and were found to be free of the cross-reactive ($a$-$d$) antibodies by gel diffusion analysis. These absorbed sera were designated "anti-$a$" and "anti-$d$", respectively.

The B13 ($d$) cells used to absorb the anti-AHT ($a$) serum were used as a source of cross-reactive ($a$-$d$) antibodies. After absorption as above, the cells were washed 3 times with cold 0.02 M sodium phosphate buffered saline (PBS) pH 7.2, suspended in 3 ml PBS, adjusted to pH 2.2 with 1 N HCl and stirred for 1 h at 4°C. The cells were pelleted at 8000 x g for 20 min. The supernate was neutralized with 1 N NaOH, dialyzed

against one liter half-strength PBS overnight and lyophilized. The residue was dissolved in one half of its original volume with deionized water. This preparation was designated "anti *a*-*d*"serum.

Quantitative precipitin analysis. Quantitative precipitin analyses were performed to determine equivalence for each antigen-antibody system that was studied. A stock solution of each antigen (ca. 50 µg/ml) was made in 0.85% saline containing 0.01 M disodium ethylenediaminetetraacetate (sEDTA), adjusted to pH 7.0 with 4 N NaOH. Antisera (20 µl anti-*d* diluted 1:2 with sEDTA, 25 µl anti-*a*, or 20 µl anti *a*-*d*) were added to 13 x 100 mm tubes. Because of the problem of insolubility of inhibitors at the higher concentrations used in the *a* antigen-anti-*a*, and the *d* antigen-anti-*d* systems, a volume of 100 µl of sEDTA (corresponding to the volume of inhibitor used) was added to the appropriate tubes, while a volume of 50 µl of sEDTA was added to the *a* antigen-anti-*a*-*d* system, and to the *d* antigen-anti-*a*-*d* system. Various dilutions of antigen (50 µl in sEDTA) were added and the tubes were incubated at 37°C for 1 h followed by incubation at 4°C for 7 days. Following incubation, 200 µl of cold sEDTA was added to each tube and the tubes were centrifuged at 1500 x g for 1 h at 4°C. The supernates were carefully decanted and the tubes were allowed to drain for 10 min onto absorbent paper. The samples were washed twice as above except with 200 µl of saline without EDTA. After thoroughly draining, the samples were dissolved in 100 µl 1 N NaOH and held for 30 min at room temperature. Folin reagent C (alkaline copper tartarate *minus* NaOH), 1.0 ml, was added and the tubes were incubated at 37°C for 10 min. Commercial Folin phenol reagent (Harleco, Gibbstown, New Jersey) was diluted 1:2 (1 N after dilution) and 100 µl was added to each tube with constant mixing. Tubes were incubated at 37°C for 30 min and read at

750 nm. A protein standard curve of rabbit gamma globulin (Calbiochem) was used to quantitate antibody protein in samples. All tubes were done in triplicate.

Quantitative precipitin inhibition. Inhibition of precipitation was performed in the same manner as the quantitative precipitin analysis except various concentrations of potential inhibitors in the above volumes of sEDTA were incubated with the antiserum for 30 min at 37°C prior to the addition of the antigen solutions. All antigen-antibody systems were reacted at the estimated equivalence point. Control tubes containing only antiserum and the appropriate volume of sEDTA were run and protein values subtracted from the readings of the experimental tubes. All inhibitions were done in triplicate.

Synthesis of methyl furanosides. Two inhibitors, α and β-methyl-D-galactofuranoside, were synthesized by the method of Augestad and Berner (1, 2). Since it was not possible to crystallize the derivatives, their concentrations were determined by the phenol-sulfuric acid assay, the solutions were evaporated to the consistancy of a syrup and then diluted to the desired concentration in sEDTA.

Chemicals. Most common salts, organic solvents and reagents, and acids were obtained from Mallinckrodt (Scientific Products, Chamblee, Georgia). All carbohydrate inhibitors were obtained from Sigma Chemical Co. (St. Louis, Missouri).

## RESULTS

Composition of purified cell walls of *S. mutans* AHT and B13. Purified cell walls of AHT and B13 were examined for chemical composition and for purity as determined by the absence of non-peptidoglycan amino acids (Table 2). The major sugars present in both walls are rhamnose, glucose and galactose in molar ratios of 3.8:1.3:1 in AHT and 7.6:4.2:1 in B13. In both cases these three sugars make up nearly 50% of the wall by dry weight. One unique feature of these walls is the presence of threonine, which is not found in the walls of other *S. mutans* serotypes. The threonine appears to be linked to lysine since upon hydrolysis with 4 N HCl at 105°C for 11 h, an unknown dipeptide peak is present in the amino acid profile; and upon employing harsher conditions (6 N HCl, 105°C, 18 h) this peak disappears, and the relative molar amounts of lysine and threonine increase with respect to the other amino acids. The presence of threonine, probably as an alanyl-threonine transpeptide was shown in the peptidoglycan of these serotypes in previous investigations (5, 6, 8, 57).

Extraction of antigens. Preliminary studies showed that antigenically reactive extracts could be obtained with mild Lancefield-type acid extractions of whole cells or cell walls, and by extraction with hot formamide. Figure 1 shows a comparison by gel diffusion of Lancefield and formamide extracts of AHT and B13 cell walls using unabsorbed, homologous sera. In both cases identity is seen between the two extracts.

Gel diffusion analysis of the formamide extracts of each strain against unabsorbed sera prepared against each organism shows a reaction

Table 2. CHEMICAL COMPOSITION OF PURIFIED CELL WALLS OF *S. mutans* AHT (*a*) and B13 (*d*)

| Major Component[a] | AHT | | B13 | |
|---|---|---|---|---|
| | µmol/mg | µg/mg | µmol/mg | µg/mg |
| Rhamnose | 1.68 | 275.9 | 1.44 | 236.1 |
| Glucose | 0.56 | 101.8 | 0.80 | 143.5 |
| Galactose | 0.44 | 78.9 | 0.19 | 33.3 |
| Glucosamine | 0.36 | 79.9 | 0.33 | 71.9 |
| Muramic Acid | 0.31 | 90.3 | 0.27 | 79.6 |
| Alanine | 1.26 | 111.9 | 1.02 | 91.1 |
| Lysine | 0.49 | 72.1 | 0.40 | 58.5 |
| Glutamic Acid | 0.45 | 66.6 | 0.41 | 60.4 |
| Threonine | 0.43 | 51.5 | 0.36 | 42.7 |
| Aspartic Acid | 0.04 | 5.6 | tr | -- |
| Glycerol | tr[b] | --- | tr | -- |
| Phosphorus | 0.18 | 5.7 | 0.31 | 9.6 |
| Total Recovery | | 940.0 | | 826.7 |

[a] Amino sugars are reported as acetylated derivatives
[b] Trace amount not measured

Figure 1. Gel immunodiffusion experiments demonstrating the reaction of identity obtained between Lancefield extracts of AHT and B13 (aLE and dLE) and formamide extracts (aFE and dFE), and the homologous, unabsorbed anti-AHT serum (Fig. 1a) and anti-B13 serum (Fig. 1b).

Fig. 1a

Fig. 1b

of partial identity in each case (Fig. 2a and 2b). Absorption of each antiserum with the heterologous strain eliminates the cross-reaction (Fig. 2c and 2d). The cross-reacting antibodies can be released from the cells used for absorption by incubation at pH 2.2 for 1 h. Figure 2e shows the reaction of antibodies released from B13 whole cells used to absorb anti-AHT serum, showing a line of identity between the two extracts.

On immunoelectrophoresis, both extracts show single bands with cathodic mobility (Fig. 3). Lancefield extracts of B13 generally show a double-arc "gull-wing" pattern, particularly when extracted for longer than 10 min (not shown here), but this and the apparent faster mobility of formamide extracts may be due to size degradation or charge alteration of the polymers as will be shown below.

For extraction of the antigens from purified cell walls, the formamide technique was used because it yielded an antigenically reactive product which was free of peptidoglycan components. Table 3 gives the compositions of the formamide extracts of both strains. The extracts are almost totally composed of carbohydrates with less than 1% phosphorus suggesting the absence of lipoteichoic acid or teichoic acid polymers as well as nucleic acid contaminants. Amino acid analysis (not shown) revealed only trace amounts of amino acids and amino sugars indicating the absence of peptidoglycan components or proteins. Routine yields of the antigenic formamide extract were about 33% of the dry weight of the cell walls used for extraction.

Purification of antigens. Initially, attempts were made to purify the antigens by gel filtration of formamide extracts. Figures 4 and 5 show the profiles of the formamide extracts on Bio-Gel P-150 which has an

Figure 2. Gel immunodiffusion experiments demonstrating the precipitin patterns obtained between the formamide (aFE or dFE) extracts and various antisera: unabsorbed anti-AHT serum (Fig. 2a), unabsorbed anti-B13 serum (Fig. 2b), anti-AHT serum absorbed with B13 cells (anti-a, Fig. 2c), anti-B13 serum absorbed with AHT cells (anti-d, Fig. 2d), and immunoglobulins released from B13 cells used to absorb anti-AHT serum (anti-a-d, Fig. 2e).

Fig. 2a

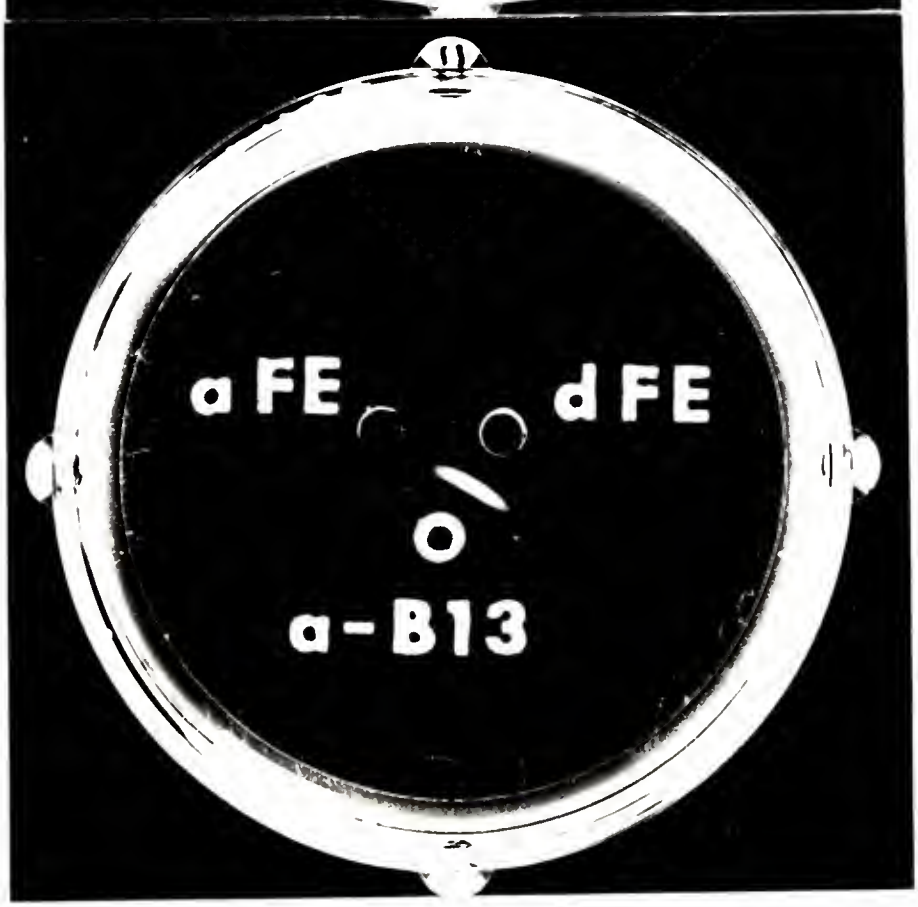



Fig. 2b

Fig. 2c

Fig. 2d

Fig. 2e

Figure 3. Immunoelectrophoretic patterns obtained with formamide (FE) and Lancefield (LE) extracts of AHT (Fig. 3a) and B13 (Fig. 3b). Troughs were filled with unabsorbed anti-AHT serum (Fig. 3a) and unabsorbed anti-B13 serum (Fig. 3b).

Fig. 3a

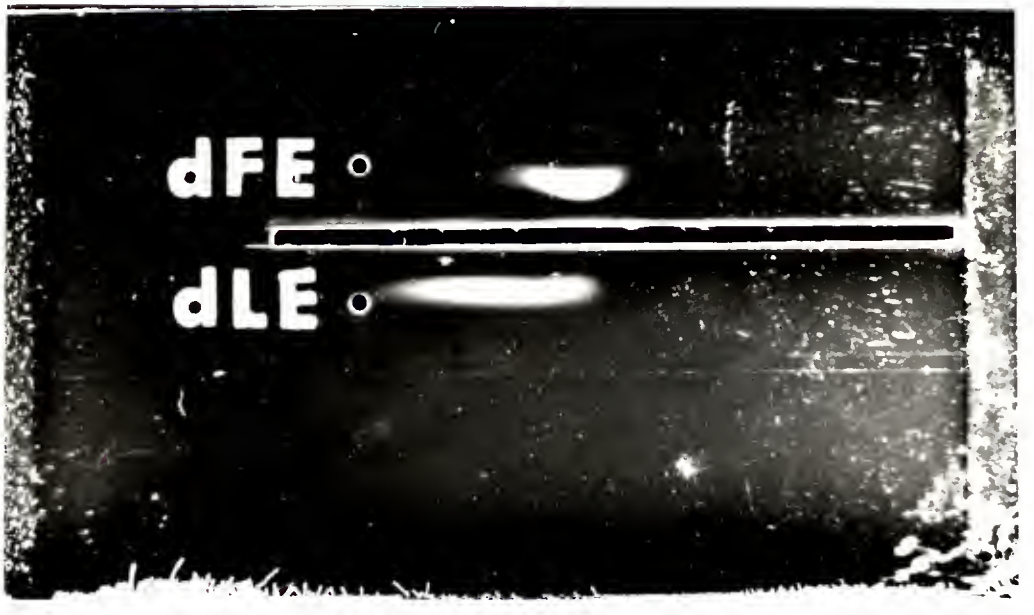



Fig. 3b

Table 3. CHEMICAL COMPOSITION OF FORMAMIDE EXTRACTS OF *S. mutans* AHT ($\alpha$) AND B13 ($d$) PURIFIED CELL WALLS

| Major Component | AHT | | B13 | |
|---|---|---|---|---|
| | µmol/mg | µg/mg | µmol/mg | µg/mg |
| Rhamnose | 3.11 | 510.4 | 3.24 | 532.2 |
| Galactose | 1.39 | 250.3 | 0.34 | 62.0 |
| Glucose | 1.08 | 193.7 | 2.05 | 369.5 |
| Phosphorus | 0.27 | 8.4 | 0.25 | 7.8 |
| Total Recovery | | 962.8 | | 971.5 |

Figure 4. Gel filtration profile of AHT formamide extract (FE, 50 mg) loaded on Biogel P-150. Fractions were assayed for total sugars ($A_{485}$), rhamnose, absorbance at 220 nm, and antigenic reactivity in capillary tubes using unabsorbed anti-AHT serum.

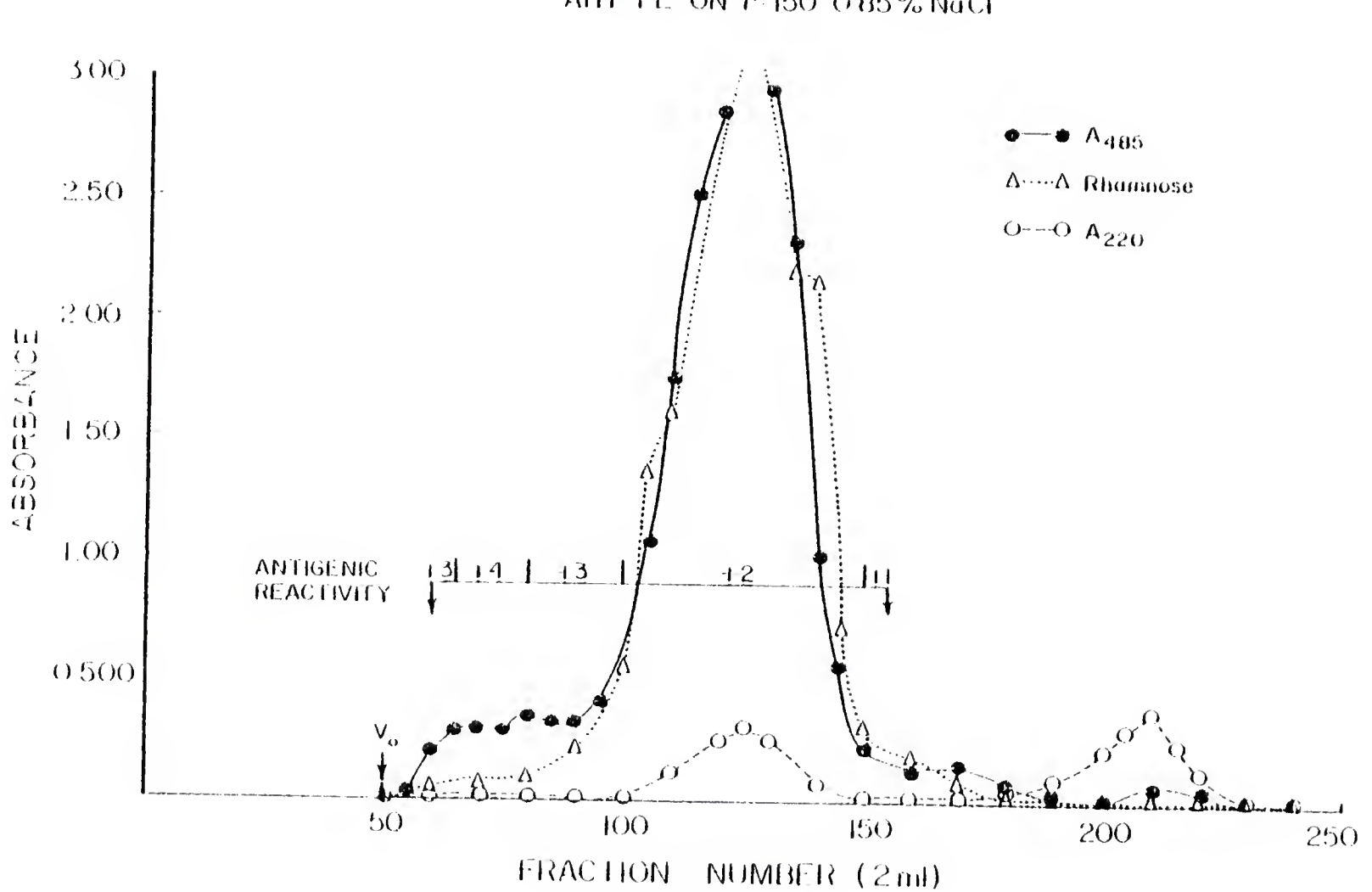
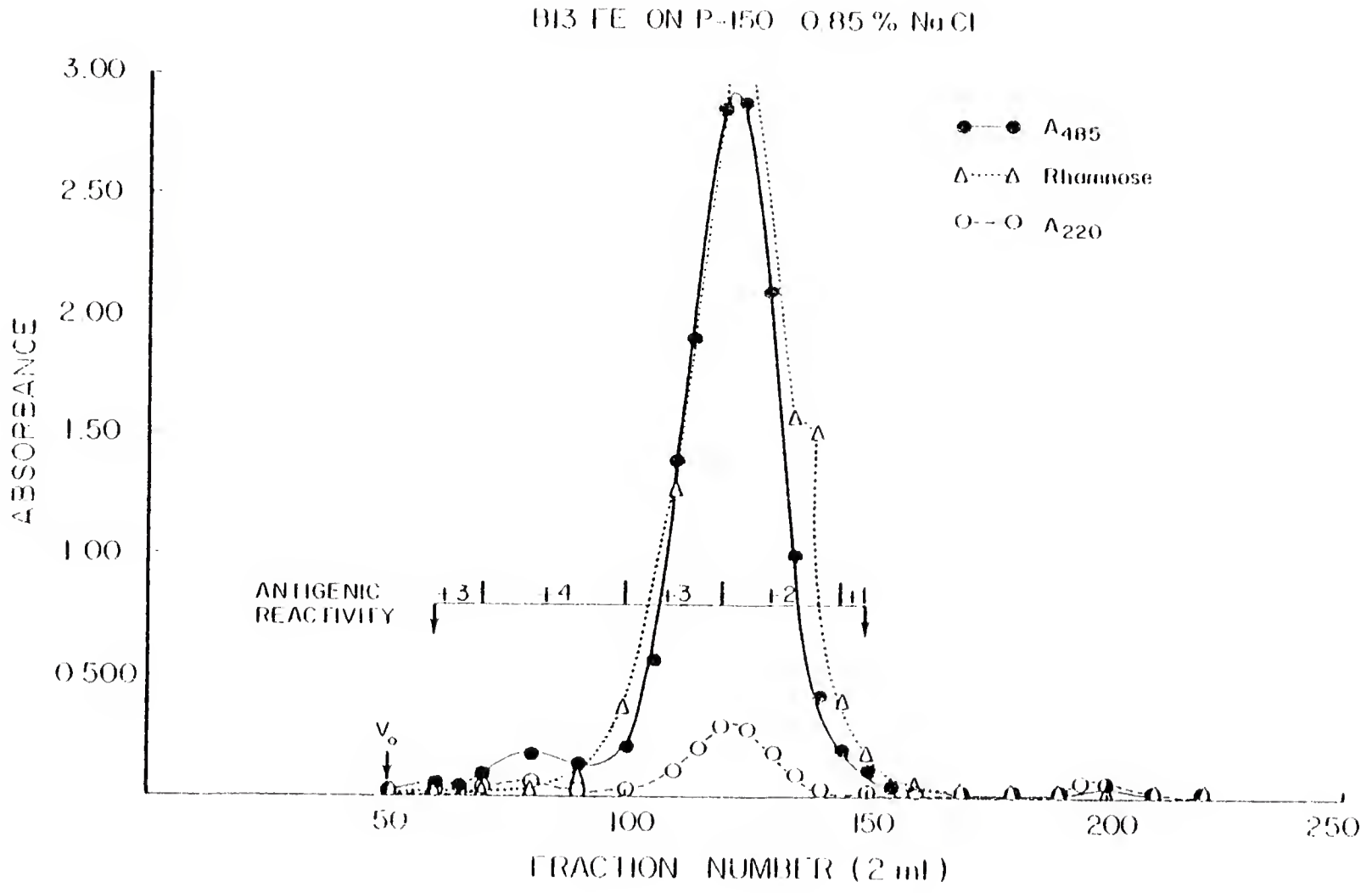

AH FE ON P-150 0.85% NaCl
3.00
2.50
2.00
1.50
1.00
0.500
ABSORBANCE
A485
Rhamnose
A220
ANTIGENIC REACTIVITY
I3
I4
I3
I2
I1
Vo
50
100
150
200
250
FRACTION NUMBER (2ml)

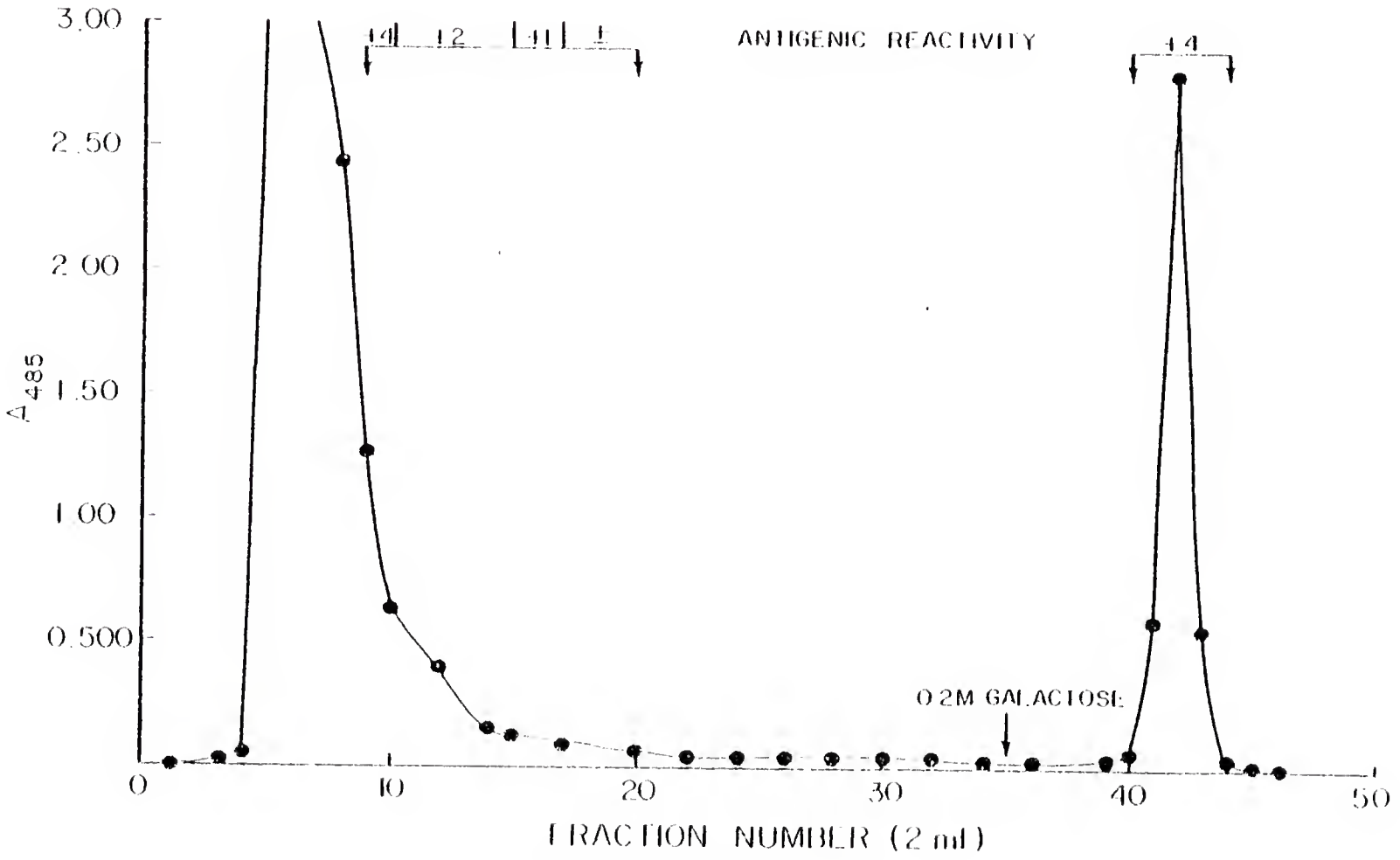
Figure 5. Gel filtration profile of B13 formamide extract (FE, 50 mg) loaded on Biogel P-150. Fractions were assayed for total sugars ($A_{485}$), rhamnose, absorbance at 220 nm, and antigenic reactivity in capillary tubes using unabsorbed anti-B13 serum.


BI3 FE ON P-150 0.85 % NaCl
3.00
2.50
2.00
1.50
1.00
0.500
ABSORBANCE
A485
Rhamnose
A220
ANTIGENIC REACTIVITY
I-3
I-4
I-3
I-2
I-1
Vo
50
100
150
200
250
FRACTION NUMBER (2 ml)

exclusion limit of 150,000 daltons. The profiles were quite similar. Fractions with antigenic reactivity emerged shortly after the void volume and trailed into the large rhamnose-containing peak. It appeared that the majority of these antigenic materials, because they emerged before the rhamnose peak, did not contain a large amount of that common methyl pentose. It was also apparent that it would be difficult to cleanly separate the antigenic materials from the non-antigenic rhamnose-containing material by gel filtration. For this reason, an affinity chromatography approach was devised employing the galactose-binding lectin from *Ricinus communis*. Initial attempts to produce an affinity support using cyanogen bromide-Sepharose 4B (Pharmacia) resulted in low capacity columns. The best results were obtained using Affi-Gel 10 (Biorad) which contains a 10 Å spacer arm. After loading 50 mg of formamide extract, the column was eluted with approximately 70 ml of buffer to ensure elution of non-binding material. Bound material was eluted using the staring buffer containing 0.2 M D-galactose and the antigen eluted as a sharp peak (Fig. 6). Results obtained using AHT crude FE (not shown) were identical to those depicted in Fig. 6. The fractions under the peaks were pooled, dialyzed against deionized water, and lyophilized. Some antigenic material passed through the column initially without binding. This is probably due to overloading of the column since these early fractions can be reloaded on the column and substantial binding occurs. Initially-unbound fractions were routinely recycled once in this manner and the bound material was pooled with the initially-bound material.

Analysis of lectin-purified antigens. Analysis of the lectin-purified antigens by gel diffusion indicates lines of complete identity between the crude formamide and Lancefield extracts, and homologous

Figure 6. Affinity chromatography column profile of B13 formamide extract (FE, 50 mg). The affinity gel (Affi-Gel 10, Biorad) contains covalently bound castor bean lectin. Unbound and some antigenic material elutes first. Bound antigenic material is eluted with the starting buffer containing 0.2 M galactose. Fractions were assayed for total sugars ($A_{485}$) and antigenic reactivity in capillary tubes using unabsorbed anti-B13 serum. Fractions collected after galactose elution were dialyzed before assaying.


AFFINITY CHROMATOGRAPHY OF B13 FE
ANTIGENIC REACTIVITY
4.4
0.2M GALACTOSE
3.00
2.50
2.00
1.50
1.00
0.500
$A_{485}$
0
10
20
30
40
50
FRACTION NUMBER (2 ml)

purified antigens of each strain (Fig. 7). Fig. 8 compares crude Lancefield and formamide extracts of AHT and B13 cell walls with the homologous lectin-purified antigen of each strain by immunoelectrophoresis. In each case a single band with the same cathodic mobility is seen. As with the formamide extracts, when the purified antigens are reacted with unabsorbed antiserum by gel diffusion, partial identity between the two antigens is seen (Fig. 9a and 9b). Absorption of the sera with the heterologous whole cells yields specific anti-$\alpha$ or anti-$\delta$ serum (Fig. 9c and 9d). Release of the immunoglobulins absorbed from anti-AHT serum by B13 cells yields a serum giving reaction of identity between the $\alpha$ and $\delta$ antigens (Fig. 9e). Gel filtration profiles of each purified antigen are shown in Figs. 10 and 11. A single, rather broad peak containing all of the antigen activity is seen for each preparation. The broadness of the peaks may reflect the range of polymer sizes which result from the rather harsh formamide extraction procedure. The average molecular weight of the B13 antigen appears lower than that for AHT and may reflect the increased susceptibility to hydrolysis of B13 cell wall polysaccharides as discussed below. Chemical analyses (Table 4) reveal that the antigens were composed primarily of galactose and glucose in a molar ratio of 3.4:1 in AHT, and 2.1:1 in B13, with only traces of rhamnose as shown by gas-liquid chromatography. Each antigen contained less than 1% phosphorus. Both antigens were quite hygroscopic, and although they were stored lyophilized and dessicated over $CaSO_4$, water of hydration collected over long periods of time and was not taken into account in data shown in Table 4. Amino acid analyses did not reveal any gross contamination with protein derived from the lectin-containing affinity column used in the purification.

Figure 7. Gel immunodiffusion experiments using crude extracts and purified antigens. In Fig. 7a, a reaction of identity is seen between AHT Lancefield extract (aLE), AHT formamide extract (aFE), and AHT purified antigen (a), using unabsorbed anti-AHT serum (center well). In Fig. 7b, identity is also seen using B13 Lancefield extract (dLE), B13 formamide extract (dFE), and B13 purified antigen (d). Center well in Fig. 7b contains unabsorbed anti-B13 serum.

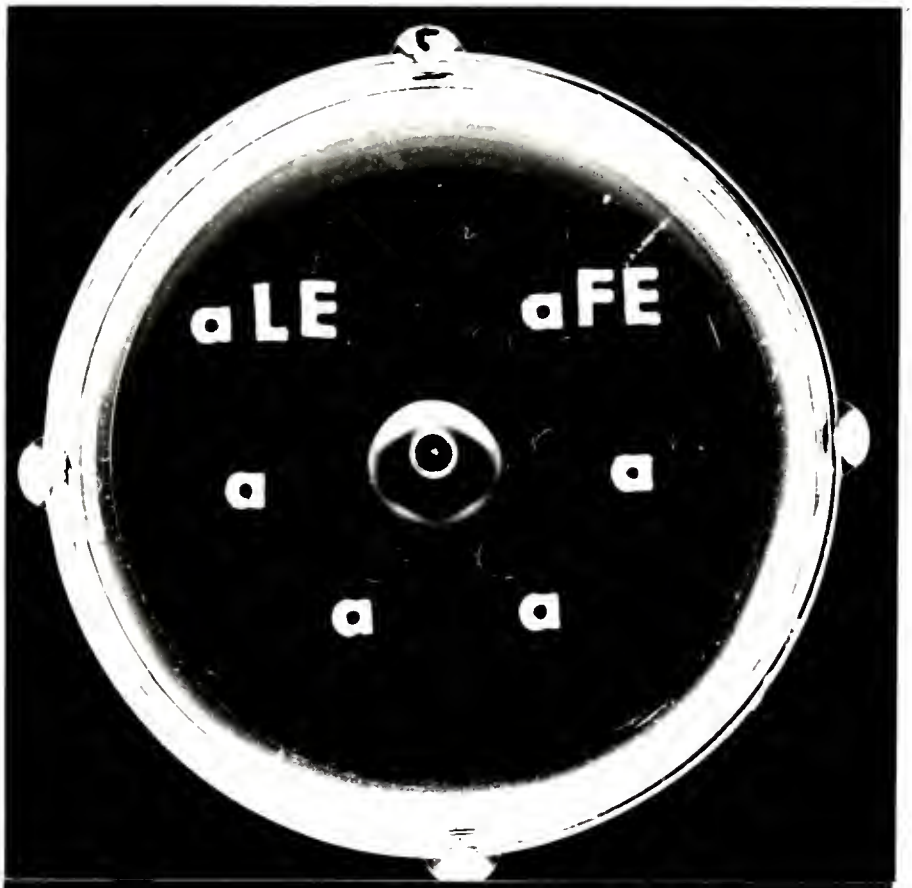



Fig. 7a

Fig. 7b

Figure 8. Immunoelectrophoretic patterns obtained with crude extracts and purified antigens. Fig. 8a: comparison of AHT formamide extract (aFE), purified antigen (a), and AHT Lancefield extract (aLE). Troughs contain unabsorbed anti-AHT serum. Fig. 8b: comparison of B13 formamide extract (dFE), purified antigen (d), and B13 Lancefield extract (dLE). Troughs contain unabsorbed anti-B13 serum.

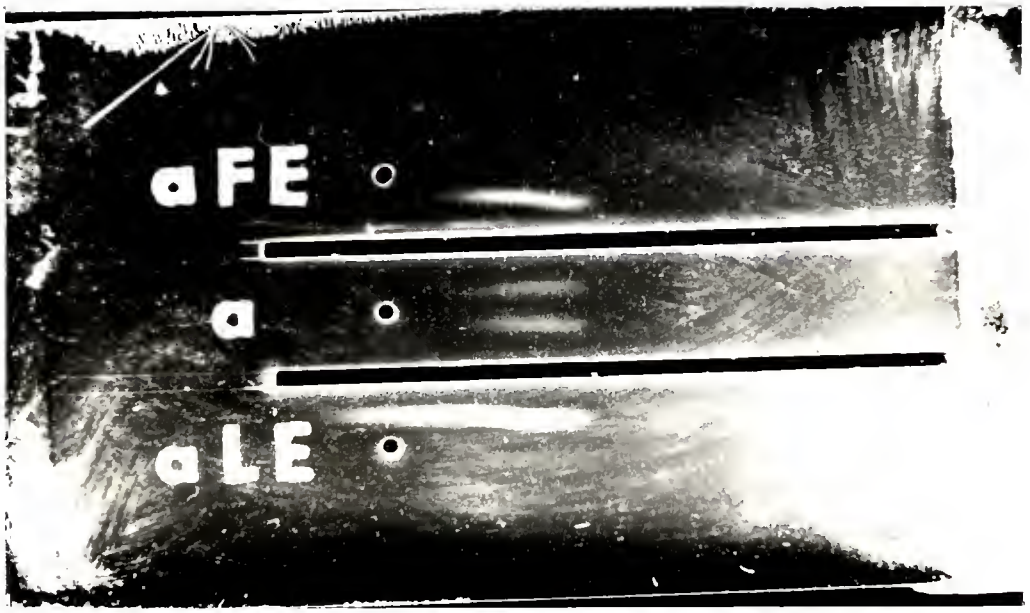



Fig. 8a

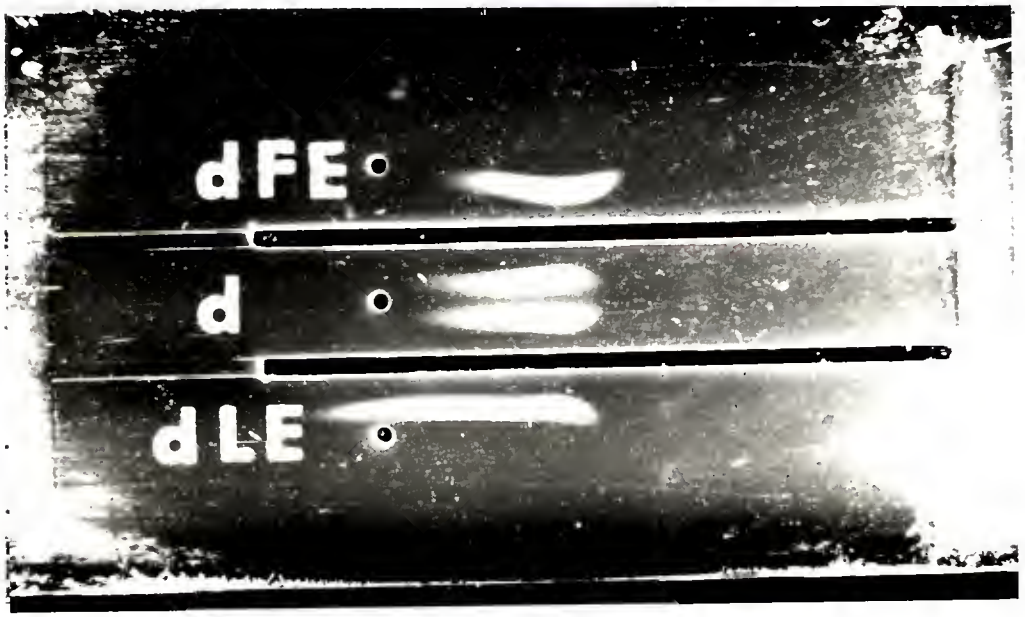



Fig. 8b

Figure 9. Gel immunodiffusion experiments using purified antigens from AHT (a) and B13 (d). Antisera used are: unabsorbed anti-AHT (a-AHT, Fig. 9a), unabsorbed anti-B13 serum (a-B13, Fig. 9b), absorbed anti-AHT serum (anti-a, Fig. 9c), absorbed anti-B13 serum (anti-d, Fig. 9d), and immunoglobulins released from B13 cells used to absorb anti-AHT serum (anti-a-d, Fig. 9e).

Fig. 9a

Fig. 9b

Fig. 9c

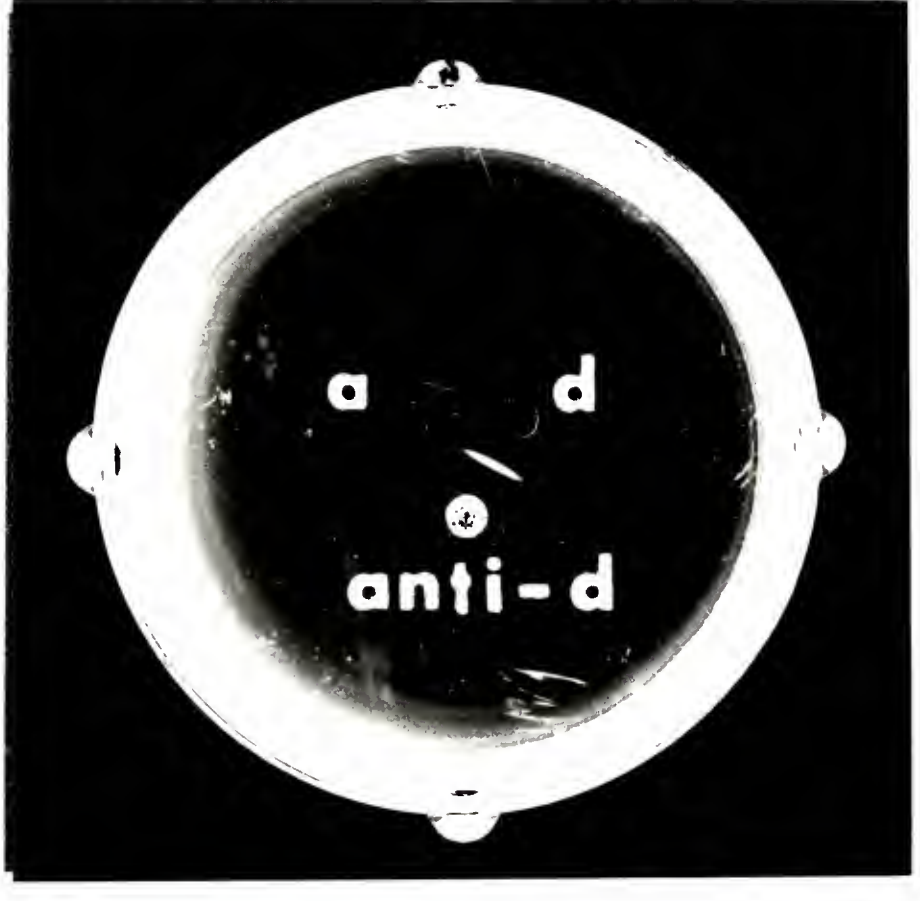



Fig. 9d

Fig. 9e

Figure 10. Gel filtration profile of AHT purified antigen (2.5 mg) on Biogel P-150. Fractions (0.2 ml) were measured for total sugars ($A_{485}$) and antigenic reactivity using capillary precipitin tubes and unabsorbed anti-AHT serum.

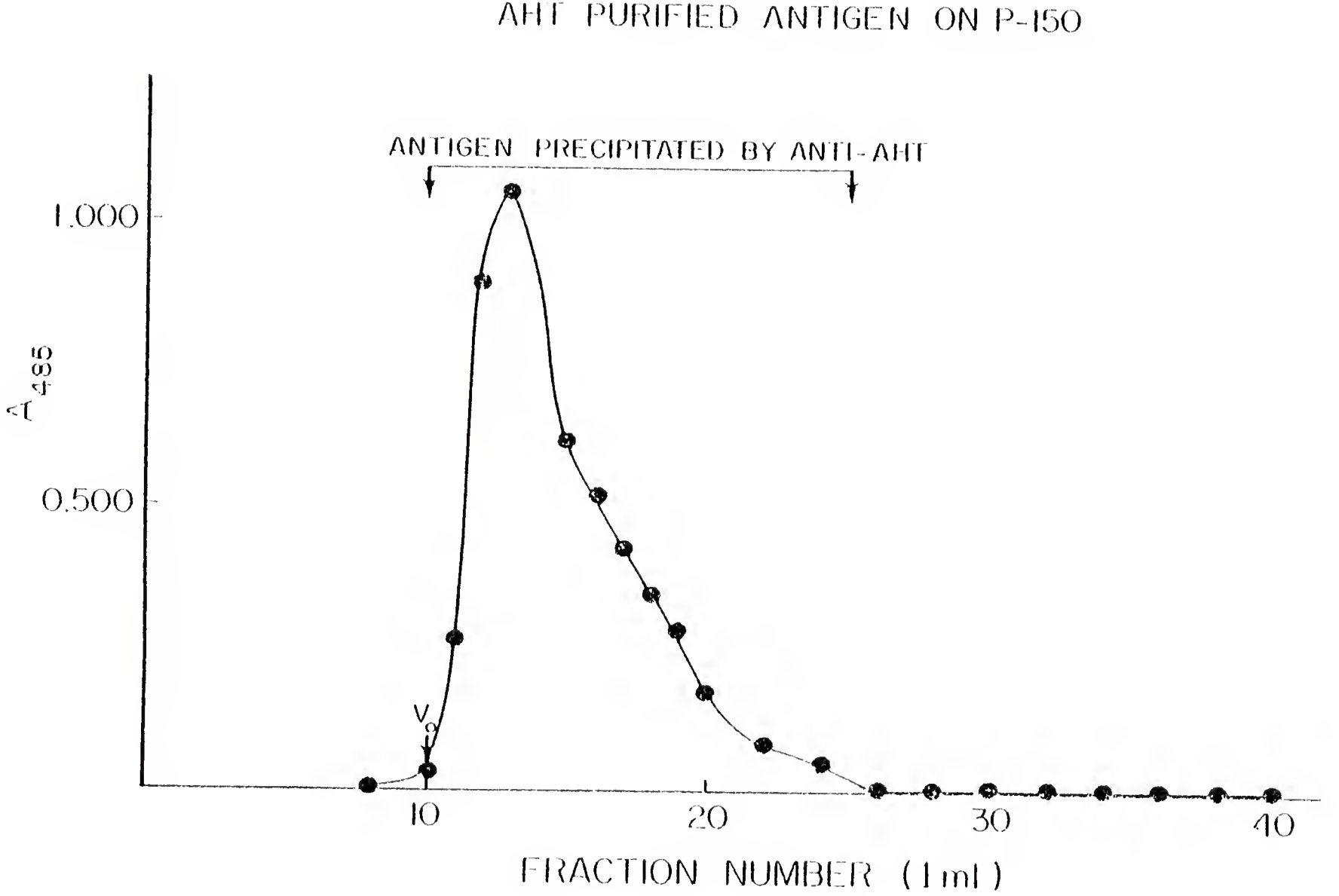

AHT PURIFIED ANTIGEN ON P-150
ANTIGEN PRECIPITATED BY ANTI-AHT
1.000
0.500
$A_{495}$
$V_o$
10
20
30
40
FRACTION NUMBER (1 ml)

Figure 11. Gel filtration profile of the B13 purified antigen (3 mg) on Biogel P-150. Fractions (0.1 ml) were assayed for total sugars ($A_{485}$) and antigenic reactivity using capillary precipitin tubes and unabsorbed anti-B13 serum.

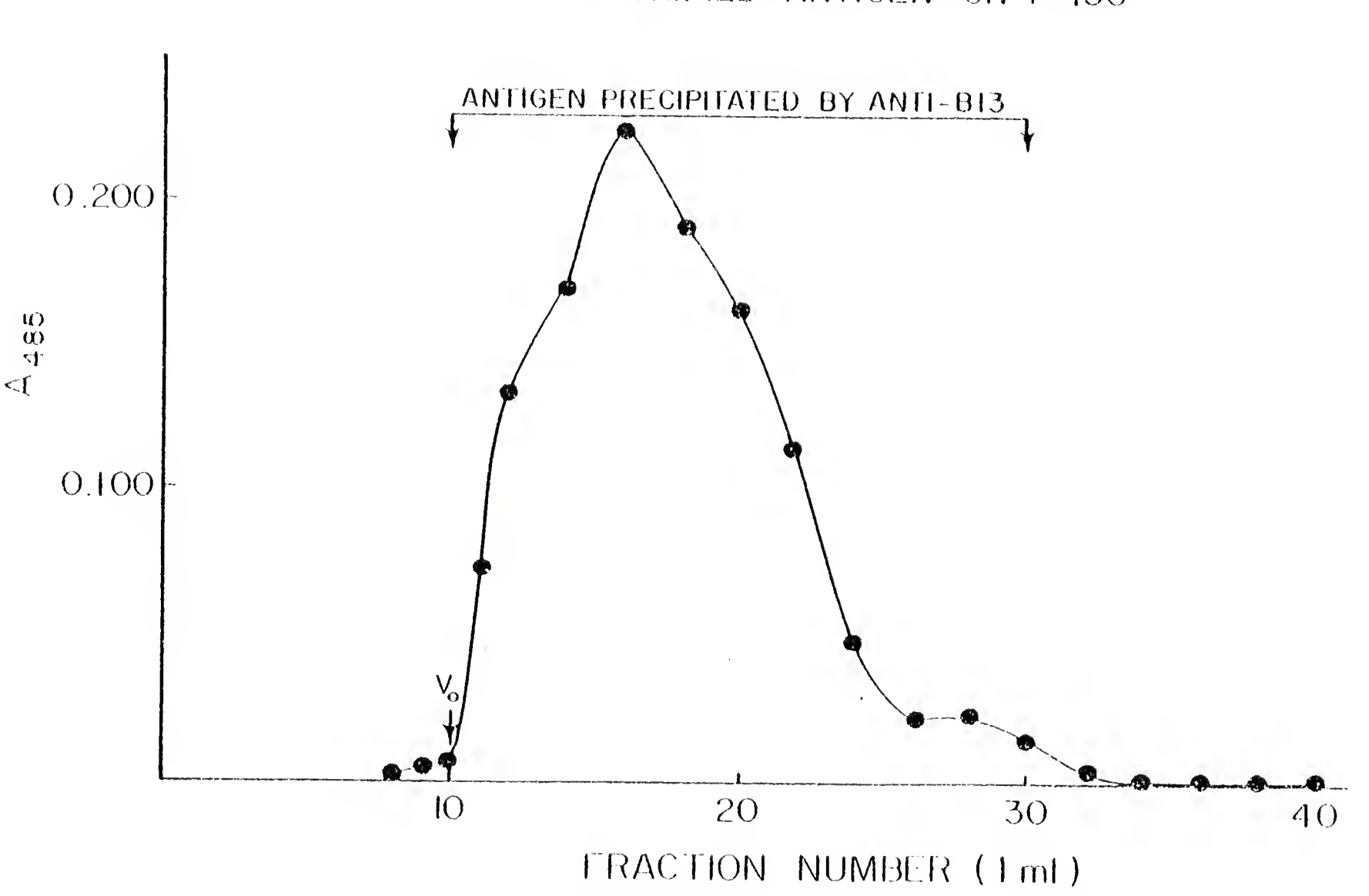

B13 PURIFIED ANTIGEN ON P-150
ANTIGEN PRECIPITATED BY ANTI-B13
0.200
0.100
A 486
$V_o$
10
20
30
40
FRACTION NUMBER (1 ml)

Table 4. COMPOSITION OF LECTIN-PURIFIED ANTIGENS FROM *S. mutans* AHT ($\alpha$) and B13 ($d$)

| Major Component[a] | AHT | | B13 | |
|---|---|---|---|---|
| | µmol/mg | µg/mg | µmol/mg | µg/mg |
| Rhamnose | tr[a] | --- | tr[a] | -- |
| Galactose | 3.34 | 601.2 | 3.33 | 599.8 |
| Glucose | 0.97 | 175.4 | 1.58 | 284.8 |
| Phosphorus | 0.28 | 8.9 | 0.22 | 7.1 |
| Total Recovery | | 785.5 | | 891.7 |

[a] Trace amount not measured

Precipitin inhibition. Inhibition of antigen-antibody precipitation was performed using absorbed sera specific for the *a* or *d* determimants of each antigen, and serum specific for the cross-reactive (or *a-d*) determinants of each antigen. The highest titers of anti-*a-d* reactivity were obtained from anti-AHT serum and this was used in the inhibition studies. Both absorbed *a-d* sera from anti-AHT or B13 showed the same immunological reactivity (Fig. 12).

A precipitin curve was run in triplicate using varying amounts of antigen and constant amounts of the absorbed antiserum (Fig. 13). For the *d*-antigen, 1.25 µg was chosen as the approximate equivalence level for studies using anti-*d* and anti-*a-d* sera, and 1.42 µg of the *a* antigen was found optimally effective for precipitation of the anti-*a* and anti-*a-d* sera.

A variety of monosaccharides, methyl glycosides and disaccharides were used as potential inhibitors. All samples and controls were run in triplicate. Table 5 shows the results of inhibition of the anti-*a* serum reaction with the purified *a* antigen. Galactose at the 50 µmol level gives the greatest inhibition (44%) *versus* 29% for glucose. The α and β-methyl glucopyranosides and galactopyranosides show no significant differences in inhibition from their non-methylated parent sugars. Of possible significance is the finding that β-methyl galactofuranoside shows greater inhibition than the α-methyl galactofuranoside, although they are both substantially lower than the corresponding galactopyranoside derivatives. Among the disaccharides tested, melibiose provides the greatest inhibition (46%) while lactose gives 39%. Both of these galactose-glucose-containing disaccharides yield greater inhibition than the three diglucosides (cellobiose, maltose, and gentiobiose), suggesting that the *a* determinant involves

Figure 12. Gel immunodiffusion experiments demonstrating the reaction of identity of cross-reacting antibodies (a-d) obtained from anti-AHT or anti-B13 sera. Top well contains the purified antigen from AHT; bottom well, the purified antigen from B13. The left well contains cross-reactive antibodies from anti-AHT serum, and the right well contains cross-reactive antibodies from anti-B13 serum.

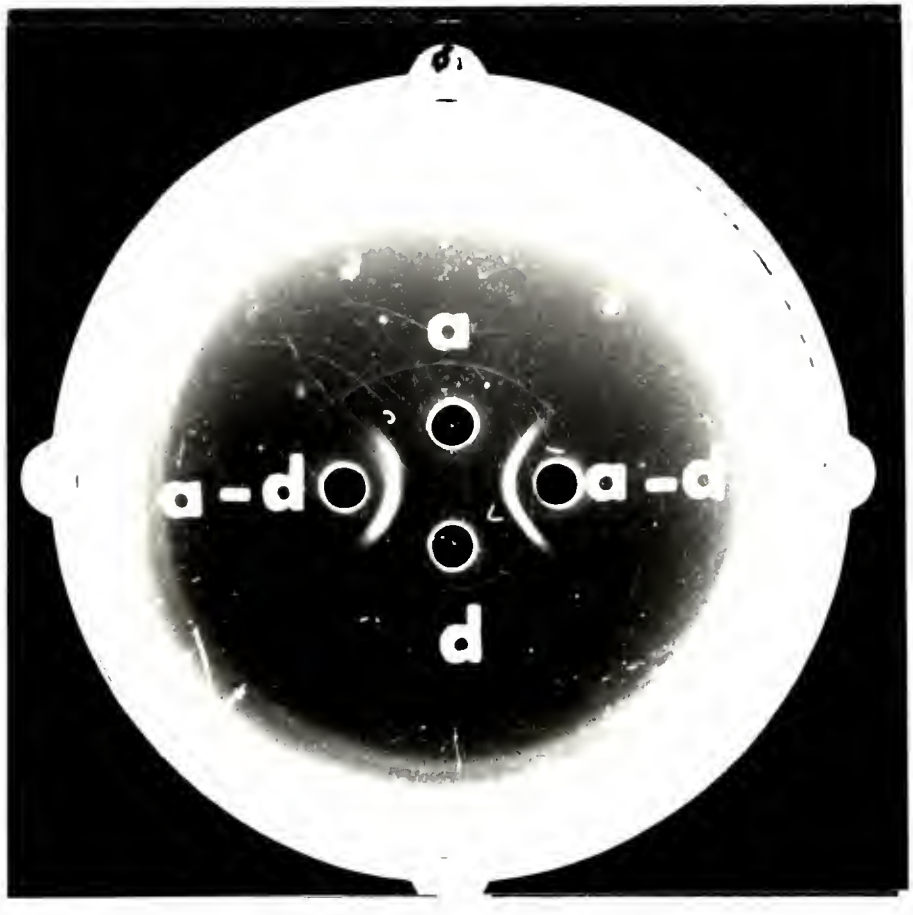



Fig. 12

Figure 13. Quantitative precipitin curves obtained using the various absorbed sera and the purified antigens.

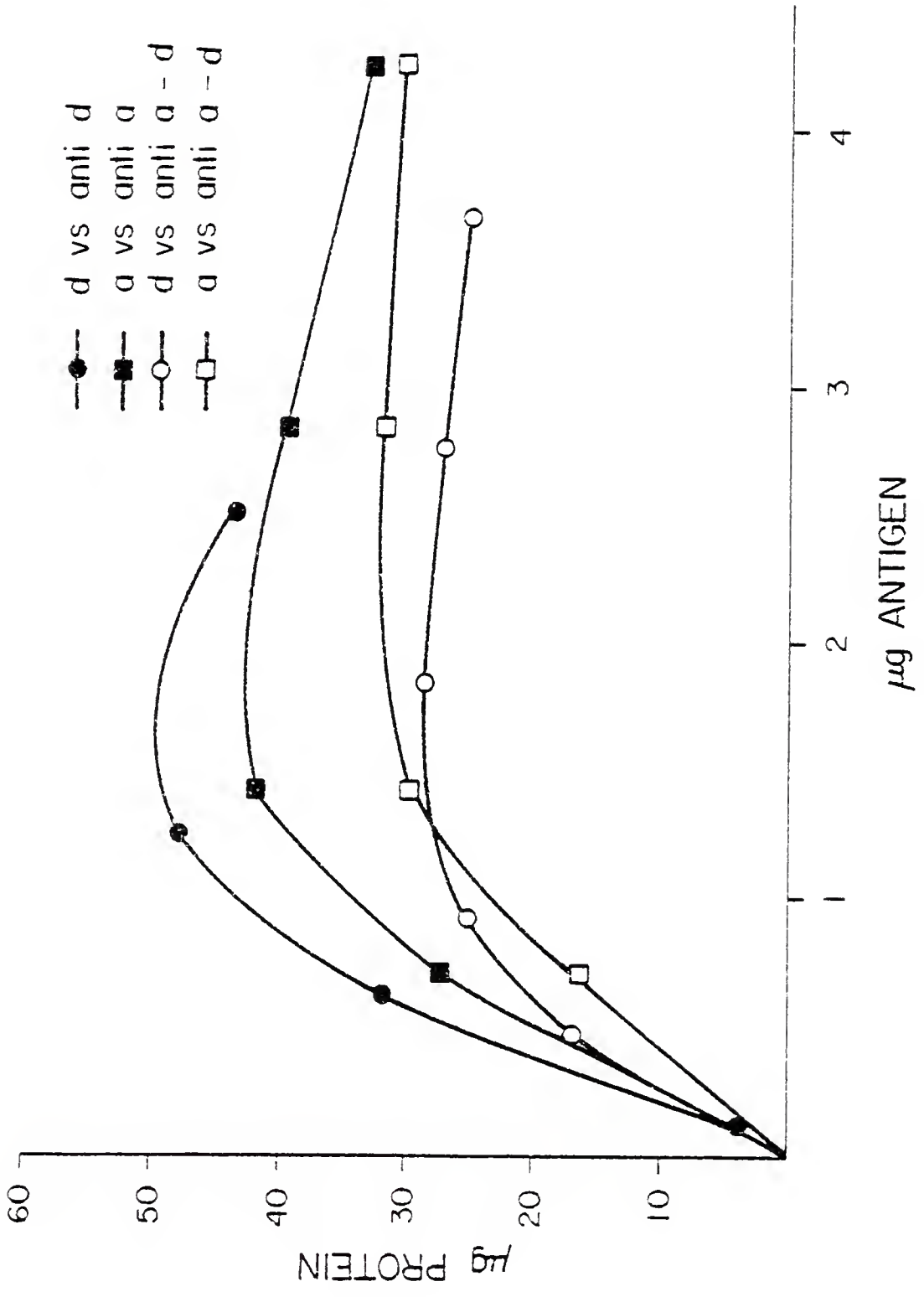

d vs anti d
a vs anti a
d vs anti a - d
a vs anti a - d
µg PROTEIN
60
50
40
30
20
10
1
2
3
4
µg ANTIGEN

Table 5. INHIBITION OF THE PRECIPITIN REACTION BETWEEN AHT ANTIGEN AND ANTI- *α* SERUM

| Inhibitor | μmol[a] | % Inhibition |
|---|---|---|
| Glucose | 50 | 29 |
| Galactose | 50 | 44 |
| α-methyl glucopyranoside | 50 | 28 |
| β-methyl glucopyranoside | 50 | 28 |
| α-methyl galactopyranoside | 50 | 46 |
| β-methyl galactopyranoside | 50 | 47 |
| α-methyl galactofuranoside | 50 | 21 |
| β-methyl galactofuranoside | 50 | 31 |
| Melibiose | 20 | 46 |
| Lactose | 20 | 39 |
| Cellobiose | 20 | 17 |
| Maltose | 20 | 21 |
| Gentiobiose | 20 | 18 |

[a]Amount in a final volume of 175μl

a galactose-glucose sequence possibly in a 1→6 linkage. (Methylation studies to be discussed below eliminate the possibility of the 1→4 linkage being present in the antigen). The anomeric nature of the linkage is not clear when one considers the results obtained with the methyl glycosides (Table 5).

Table 6 shows the results of inhibition of the anti-*d* serum reaction with the purified *d* antigen. In this case the results are not nearly as clear as for the *a*-anti-*a* system. At the 100 μmole level, glucose provides 59% inhibition of precipitation while galactose yields 47% inhibition. The β-methyl glucopyranoside derivative gives nearly twice the inhibition provided by the α-derivative, while both galactopyranosides give about the same inhibition as the parent sugar, D-galactose. The galactofuranosides are considerably less effective. All of the disaccharides provide substantial inhibition although the two diglucosides, maltose and gentiobiose, allow slightly higher inhibition, 53% and 50% respectively. Cellobiose was insoluble at the 40 μmole level, however, at the 20 μmole level it gave slightly less inhibition than the other two diglucosides at the lower level. It appears that a glucose-glucose sequence or a glucose-galactose sequence is involved in the *d* antigen specificity. However, it is not possible to establish clearer indications of the nature of the *d*-specific hapten, including linkage patterns due to the relative insensitivity of specific antibody in these inhibition studies. However, as also the case for the *a* antigen, 1→4 linkages (as in maltose) are absent (as shown by methylation studies to be presented below) and thus could not contribute to the specificity of the *d*-reactive site.

Table 6. INHIBITION OF THE PRECIPITIN REACTION BETWEEN B13 ANTIGEN AND ANTI- *d* SERUM

| Inhibitor | µmol[a] | % Inhibition |
|---|---|---|
| Glucose | 50 | 36 |
| | 100 | 59 |
| Galactose | 50 | 27 |
| | 100 | 47 |
| α-methyl glucopyranoside | 50 | 11 |
| | 100 | 25 |
| β-methyl glucopyranoside | 50 | 24 |
| | 100 | 48 |
| α-methyl galactopyranoside | 50 | 20 |
| | 100 | 44 |
| β-methyl galactopyranoside | 50 | 15 |
| | 100 | 46 |
| α-methyl galactofuranoside | 50 | 19 |
| β-methyl galactofuranoside | 50 | 18 |
| Melibiose | 20 | 28 |
| | 40 | 44 |
| Lactose | 20 | 25 |
| | 40 | 46 |
| Cellobiose | 20 | 27 |
| | 40 | i[b] |
| Maltose | 20 | 31 |
| | 40 | 53 |
| Gentiobiose | 20 | 30 |
| | 40 | 50 |

[a]Amount in a final volume of 170µl
[b]Insoluble

The results of the *a-d* specificity studies for both antigens were quite similar, as would be expected (Tables 7 and 8). The general trends are the same in both cases. Galactose gives greater inhibition than glucose. The two 1→6 linked disaccharides, melibiose and gentiobiose, allow the greatest inhibition. No conclusions concerning anomeric linkage can be drawn from the use of methylated derivatives of glucose or galactose since the inhibitions provided are quite similar. From these data (Tables 7 and 8) one can only conclude that galactose plays a major role in the *a-d* specificity and a 1→6 linkage may be involved.

Some of the conclusions derived from these precipitin inhibition studies are at variance with those recently published for strain B13 (*d*) (42) and another serotype *a* organism, strain HS6 (46). These differences will be presented below in the *Discussion*.

Methylation analysis. Results of the methylation analysis of the purified antigens are shown in Tables 9 and 10. These data give us specific information on the types of linkages in which all constituent sugars are involved, and the relative frequencies of each linkage type in each polysaccharide.

To determine the exact position of each residue in the polymer would necessitate isolation and characterization of several disaccharides and oligosaccharides produced by partial hydrolysis of the antigenic polysaccharide. The large amounts of material required prevented us from taking this classical approach: however, much information on polymeric structure can be gained from methylation analysis alone.

In each polymer there are four residues in identical molar ratios with respect to the branch-point. Methylation of each polymer yields 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol (2,3,4,6 Gal),

Table 7. INHIBITION OF THE PRECIPITIN REACTION BETWEEN AHT ANTIGEN AND ANTI- *a-d* SERUM

| Inhibitor | μmol[a] | % Inhibition |
|---|---|---|
| Glucose | 25 | 21 |
| Galactose | 25 | 38 |
| α-methyl glucopyranoside | 25 | 26 |
| β-methyl glucopyranoside | 25 | 23 |
| α-methyl galactopyranoside | 25 | 29 |
| β-methyl galactopyranoside | 25 | 23 |
| α-methyl galactofuranoside | 25 | 18 |
| β-methyl galactofuranoside | 25 | 4 |
| Melibiose | 10 | 47 |
| Lactose | 10 | 11 |
| Cellobiose | 10 | 33 |
| Maltose | 10 | 24 |
| Gentiobiose | 10 | 47 |

[a]Amount in a final volume of 120μl

Table 8. INHIBITION OF THE PRECIPITIN REACTION BETWEEN B13 ANTIGEN AND ANTI- *a-d* SERUM

| Inhibitor | μmol[a] | % Inhibition |
|---|---|---|
| Glucose | 25 | 29 |
| Galactose | 25 | 38 |
| α-methyl glucopyranoside | 25 | 17 |
| β-methyl glucopyranoside | 25 | 31 |
| α-methyl galactopyranoside | 25 | 25 |
| β-methyl galactopyranoside | 25 | 30 |
| α-methyl galactofuranoside | 25 | 26 |
| β-methyl galactofuranoside | 25 | 11 |
| Melibiose | 10 | 72 |
| Lactose | 10 | 16 |
| Cellobiose | 10 | 25 |
| Maltose | 10 | 41 |
| Gentiobiose | 10 | 56 |

[a]Amount in a final volume of 120ul

Table 9. METHYLATION ANALYSIS OF THE AHT ANTIGEN

| DERIVATIVE | RELATIVE AREA | RRT[a] | $RRT_{lit}$[b] | | STRUCTURE |
|---|---|---|---|---|---|
| 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol | [1.0] | 6.06[c] | 6.35[c] | | $\overset{\vert 3}{-^{6}\mathrm{Gal}^{1}\rightarrow}$ |
| <u>OR</u> | | | | | |
| 1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl-D-galactitol | | | | <u>OR</u> | $\overset{\vert 2}{-^{6}\mathrm{Gal}f^{1}\rightarrow}$ |
| 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol | 1.0 | 1.21 | 1.19 | | $\mathrm{Gal}^{1}\rightarrow$ |
| 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol | 1.8 | 2.18 | 2.22 | | $-^{6}\mathrm{Glu}^{1}\rightarrow$ |
| 1,5,6-tri-*O*-acetyl-2.3.4-tri-*O*-methyl-D-galactitol | 1.4 | 2.90 | 2.89 | | $-^{6}\mathrm{Gal}^{1}\rightarrow$ |
| 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol | 1.9 | 2.01 | 2.03 | | $-^{3}\mathrm{Gal}^{1}\rightarrow$ |
| 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol | 0.4 | 1.0 | 1.0 | | $\mathrm{Glu}^{1}\rightarrow$ |
| 1,3,4-tri-*O*-acetyl-2,5,6-tri-*O*-methyl-D-glactitol | 1.5 | 1.94 | 1.95 | | $-^{3}\mathrm{Gal}f^{1}\rightarrow$ |

[a]Relative retention time on OV-225 column by gas-liquid chromatography
[b]Literature value
[c]Relative retention time on ECNSS-M column by gas-liquid chromatography

<u>Note</u>: Gal*f* = galactofuranose; Gal = galactopyranose; Glu = glucopyranose

Table 10. METHYLATION ANALYSIS OF THE B13 ANTIGEN

| DERIVATIVE | RELATIVE AREA | RRT[a] | $RRT_{lit}$[b] | | STRUCTURE |
|---|---|---|---|---|---|
| 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol | [1.0] | 6.10[c] | 6.35[c] | | $\underline{6}\mathrm{Gal}\overset{3}{\rightarrow}\overset{1}{\rightarrow}$ |
| OR | | | | | |
| 1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl-D-galactitol | | | | OR | $\underline{6}\mathrm{Gal}f\overset{2}{\rightarrow}\overset{1}{\rightarrow}$ |
| 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol | 1.1 | 1.2 | 1.19 | | $\mathrm{Gal}\overset{1}{\rightarrow}$ |
| 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol | 1.8 | 2.18 | 2.22 | | $\underline{6}\mathrm{Glu}\overset{1}{\rightarrow}$ |
| 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol | 1.4 | 2.88 | 2.89 | | $\underline{6}\mathrm{Gal}\overset{1}{\rightarrow}$ |
| 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol | 1.5 | 1.79 | 1.82 | | $\underline{3}\mathrm{Glu}\overset{1}{\rightarrow}$ |
| 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*-methyl-D-galactitol | 0.5 | 2.76 | 2.76 | | $\underline{6}\mathrm{Gal}f\overset{1}{\rightarrow}$ |

[a]Relative retention time on OV-225 column by gas-liquid chromatography
[b]Literature value
[c]Relative retention time on ECNSS-M column by gas-liquid chromatography

Note: Gal*f* = galactofuranose; Gal = galactopyranose; Glu = glucopyranose

representing a terminal galactose residue, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol (2,3,4 Glu), representing glucose linked at C-1 and C-6, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol (2,3,4 Gal) which represents a galactose residue linked at C-1 and C-6. Finally, both polymers yield the same branch-point derivative which has been identified as either 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-galactitol (2,4 Gal) *or* 1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl-D-galactitol (3,5 Gal). These two derivatives are actually identical since they are symmetrical. This derivative is obtained from the methylation of a galactopyranose residue linked at C-1,3, and 6 or a galactofuranose residue linked at C-1,2, and 6. These two particular galactose residues yield the same derivative when methylated and reduced and can only be differentiated by tagging C-1 by reduction with sodium borodeuteride.

The four residues mentioned above may comprise a common sequence of sugars in each polymer. It will be postulated that the terminal galactose residues attached at the galactose branch-point compose part or all of the *a-d* reactive site since these residues are common to both polymers, and inhibition studies indicate that alactose may be the principal determinant of *a-d* specificity (*see Discussion*).

There are two 1→3 linked moieties in each polymer in nearly equal molar ratios with respect to the branch-point: a 1→3 linked galactose residue in the AHT polymer (1.9:1), and 1→3 linked glucose in the B13 polymer (1.5:1). The 1→3 linkage of these residues may produce pronounced "buckling" of the polymeric chain allowing these and neighboring residues to be presented as a sterically-accessible antigenic unit to antibody molecules. It will be proposed (*see Discussion*) that these as well as neighboring residues constitute the *a* and *d* determinants in the respective polymers. The AHT polymer

contains the 1→3 linked galactose residue and inhibition studies described above show that a galactose-glucose sequence gives the greatest inhibition, while the B13 polymer contains the 1→3 linked glucose residue and inhibition studies suggest a glucose-glucose sequence is involved.

Another feature of both polymers that becomes evident from the methylation studies is the presence of galactofuranose residues. Methylation of the AHT polymer yields 1,3,4-tri-*O*-acetyl-2,5,6-tri-*O*- methyl galactitol (2,5,6 Gal) which represents a galactofuranose residue linked at C-1 and 3 to neighboring sugars. The B13 polymer yields 1,4,6-tri-*O*-acetyl-2,3,5-tri-*O*- methyl galactitol (2,3,5 Gal) upon methylation, which represents a galactofuranose residue linked at C-1 and 6 in the native polymer. Furanosidic linkages are known to be much more sensitive to acid hydrolysis than the corresponding linkages between pyranosides (3,50,52).

A study using immunoelectrophoresis was undertaken to demonstrate the acid lability of these polymers and to compare the apparent greater mobility of formamide extracts with products yielded by Lancefield acid extractions of B13. Samples of B13 cell walls were hydrolyzed at pH 2.5 according to the procedure outlined in *Methods*, except that samples were hydrolyzed for 10,20,40 or 60 min before neutralization. Fig. 14a and b show the results of the hydrolysis of B13 cell walls at pH 2.5. After 10 min, essentially a single arc is seen. With increasing hydrolysis times, however, a "gull-wing" type of precipitin band is obtained with the faster-moving portion of the band becoming more evident and possessing a mobility similar to that of the formamide extract shown in the center well. To determine whether this represents the degradation of the polymer or the extraction of a second antigen from the cell wall, the supernatant

Figure 14. Immunoelectrophoretic study of the degradation of Lancefield extracts of B13 cell walls at various extraction times. In Fig. 14a and Fig. 14b, cell walls were extracted for 10,20,40, and 60 min and compared to a formamide extract (FE). In Fig. 14c the supernate from a 10 min Lancefield extract was hydrolyzed for an additional 30 min (10 + 30). In Fig. 14d the supernate from a 10 min Lancefield extract was subjected to formamide extraction (FE of LE). Unabsorbed anti-B13 serum was used in all troughs.

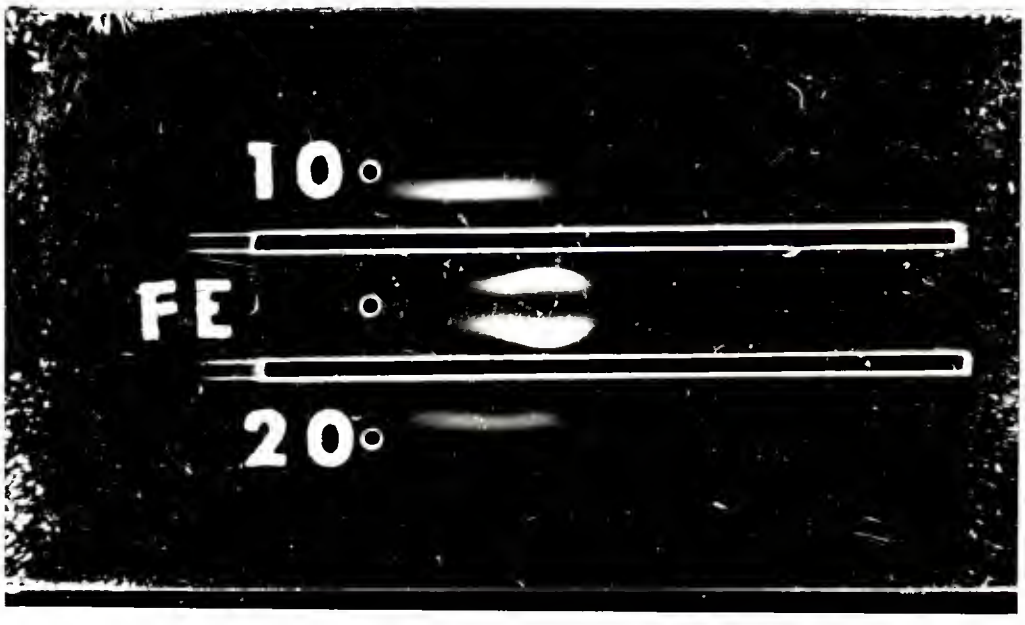



Fig. 14a

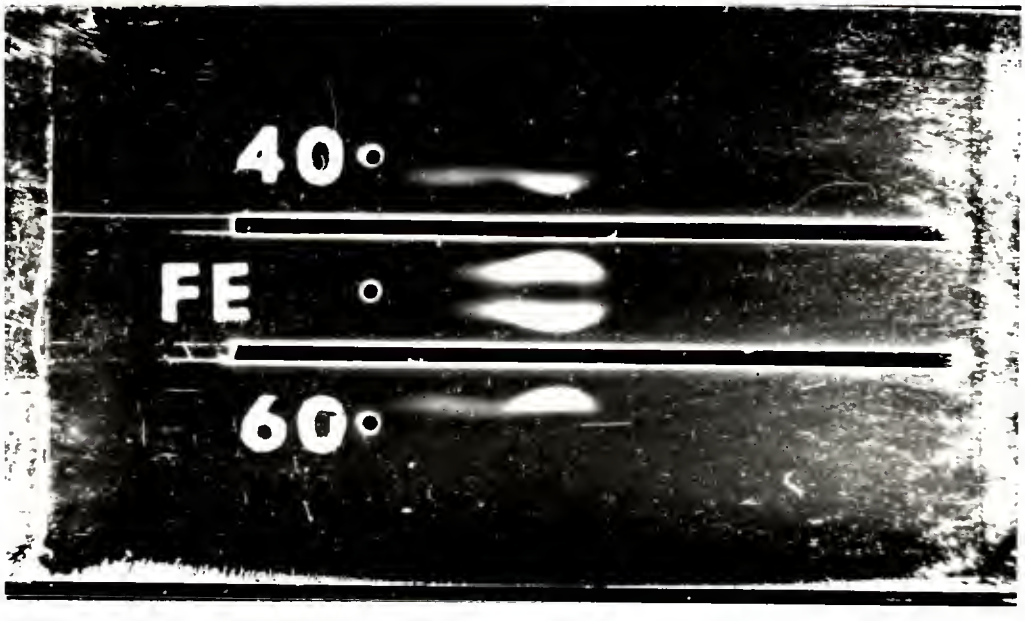



Fig. 14b

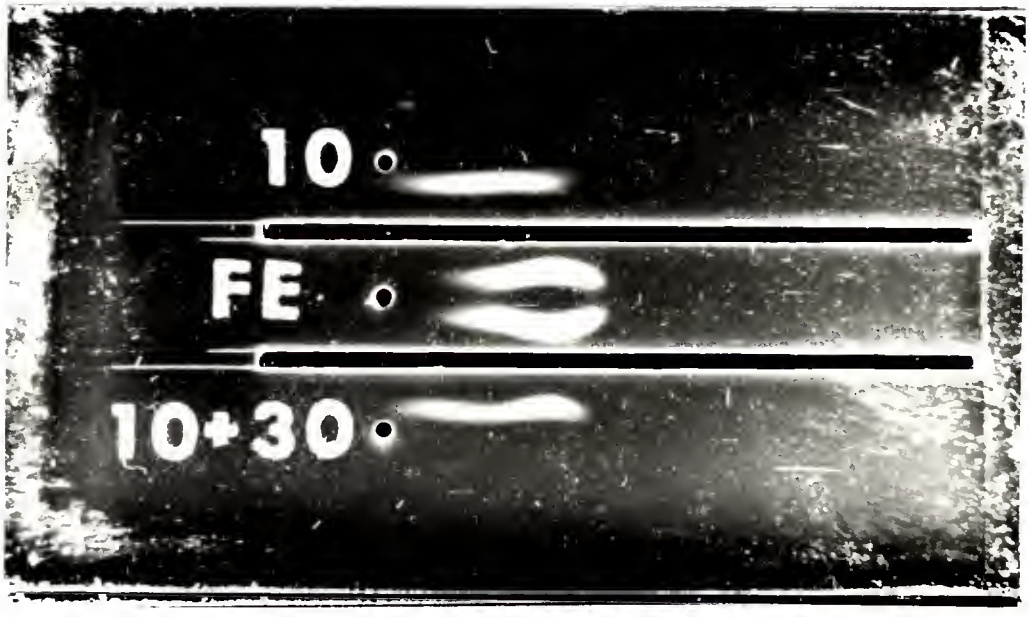



Fig. 14c

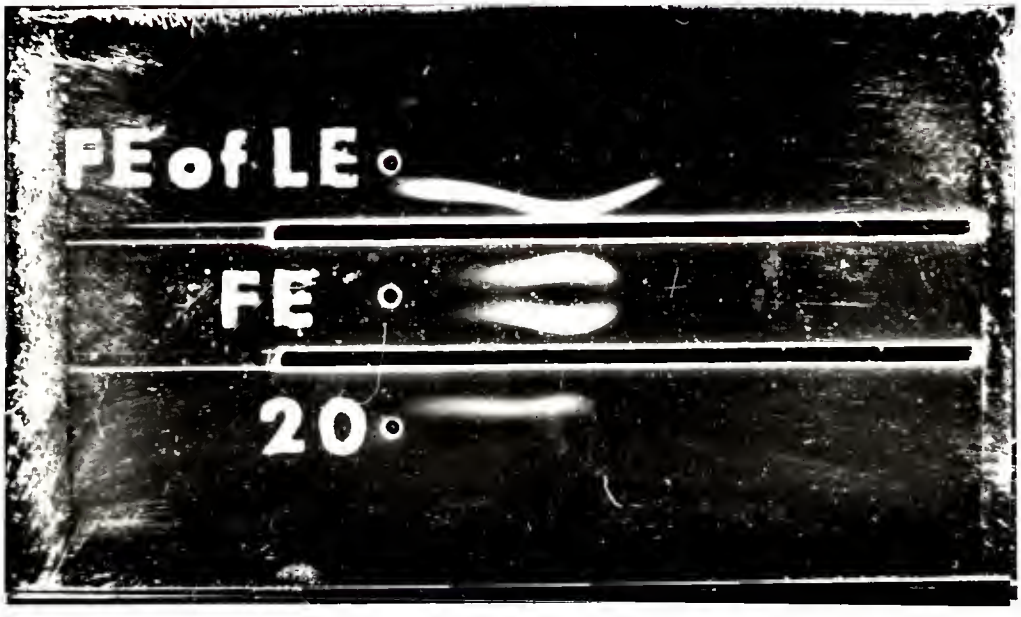



Fig. 14d

from the 10 min extract which had been neutralized and centrifuged to remove cell walls was readjusted to pH 2.5 and boiled for an additional 30 min. The appearance of the familiar "gull-wing" pattern upon additional hydrolysis (Fig. 14c) suggests that this phenomenon is likely due to size degradation or an alteration of the charge of the polymer and not due to extraction of a second antigen from the wall. Likewise, if the neutralized 10 min supernatant is subjected to formamide extraction (Fig. 14d), further degradation of the polymer is apparent.

The same type of experiment was repeated using AHT walls (Fig. 15). Again, degradation can be seen with increasing hydrolysis time. When the neutralized, cell wall-free supernatant of the 10 min acid extract is subjected to formamide extraction, the conversion to a faster moving component is seen as in the case of B13 (Fig. 15c).

Figure 15. Repeat of the experiment shown in Fig. 14 using AHT Lancefield cell wall extracts (10,20,40, and 60 min) and AHT formamide extract (FE). Time degradation of AHT antigen showing the appearance of a faster moving component (Fig. 15a and Fig. 15b). Supernate from a 10 min Lancefield extract was further treated by formamide extraction (FE of LE) and compared to a formamide extract of cell walls (FE) and a 10 min Lancefield extract (LE, Fig. 15c). All troughs were filled with unabsorbed anti-AHT serum.

Fig. 15a

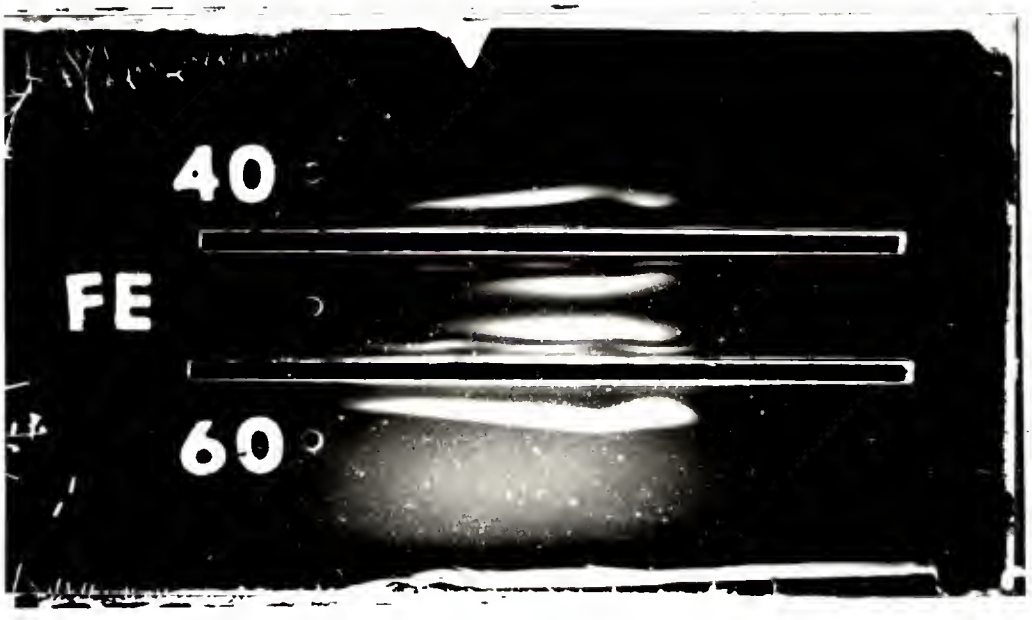



Fig. 15b

Fig. 15c

# DISCUSSION

Two antigenic polysaccharides were extracted and purified from cell walls of the cross-reactive strains *S. mutans* AHT *(a)* and *S. mutans* B13 *(d)*. The antigens, extracted by the hot formamide method, were purified by affinity chromatography on columns containing the galactose-specific lectin from the castor bean (ricin) and found to be diheteroglycans consisting primarily of galactose and glucose. Antigenic specificities of both the specific and the cross-reactive sites on each polymer were determined, and methylation analysis revealed structural similarities that may reflect the nature of the cross-reactive sites and differences that may reflect the natures of specific haptenic regions.

At the present time there are only three papers dealing with the immunochemistry of these two serotypes (41, 42, 46). Two points must be considered when comparing the present study with the previously published data: first, the starting materials (cell walls *vs* whole cells) and the methods of extraction and purification will undoubtedly affect not only the size of the polymer extracted but also the amounts and types of materials that may be associated with the purified products (peptidoglycans, lipoteichoic acids, etc.) but which do not contribute to the antigenic reactivity of the product. These differences should not, however, affect the specificity of the combining sites with respect to antibodies when measured by precipitin inhibition assays. Second, one is limited by the availability of appropriate potential inhibitors - particularly disaccharides and higher oligosaccharides - for use in precipitin inhibition studies.

The results of the inhibition of precipitation of the AHT antigen with anti-$\alpha$ serum show that galactose is the best monosaccharide inhibitor (44%), with melibiose and lactose also giving relatively high degrees of inhibition. Since methylation analysis indicates the absence of 1→4 linkages in the purified AHT antigen, the inhibition by lactose is probably not a function of its linkage pattern. But the presence of 1→6 linkages in the antigen may explain the efficacy of melibiose in the inhibition (46%) of the homologous reaction. Both disaccharides, however, contain galactose which undoubtedly is located at the $\alpha$-specific haptenic site. The use of methylated galactopyranosides and galactofuranosides in inhibition studies failed to reveal the probable anomeric linkages of galactose moieties at the antibody binding site.

The $\alpha$ antigen isolated by Mukasa and Slade (46) from hot water extracts of whole cells of HS6, differs in chemical composition from the AHT antigen in the molar ratio of galactose to glucose (5.2:1 for HS6 (26) *vs* 3.4:1 for AHT) but appears to behave similarly in gel diffusion and immunoelectrophoresis. Each product apparently possesses both an $\alpha$ and $\alpha$-$\bar{d}$ reactive site. Inhibition studies on the $\alpha$ site of the HS6 polysaccharide were carried out by Mukasa and Slade (46) with only a limited number of inhibitors. Significant inhibition was obtained with glucose (25%), and the diglucosides maltose (51%), and gentiobiose (49%). The only galactose-containing inhibitor used, lactose, yielded less than 10% inhibition. All inhibitors were compared on an equal weight rather than equimolar basis. From these limited data they concluded that either α-1→4 or a β-1→6 configuration involving only glucose was responsible for the $\alpha$ specificity in HS6. Their failure to employ galactose or galactosides in their studies makes interpretation of their findings difficult.

Certainly, the present study of the AHT antigen fails to confirm the immunochemical nature of the serotype *a* antigen presented by Mukasa and Slade (46). Confidence in data presented in this dissertation is provided by the method of antigen extraction (purified cell walls rather than whole cells), the process of antigen purification (affinity chromatography) and the wider range of monosaccharides and disaccharides tested in precipitin inhibition analyses. It would appear, therefore, that further studies of serotype *a* polysaccharides may be required to present a more coherent picture of this important antigenic polysaccharide.

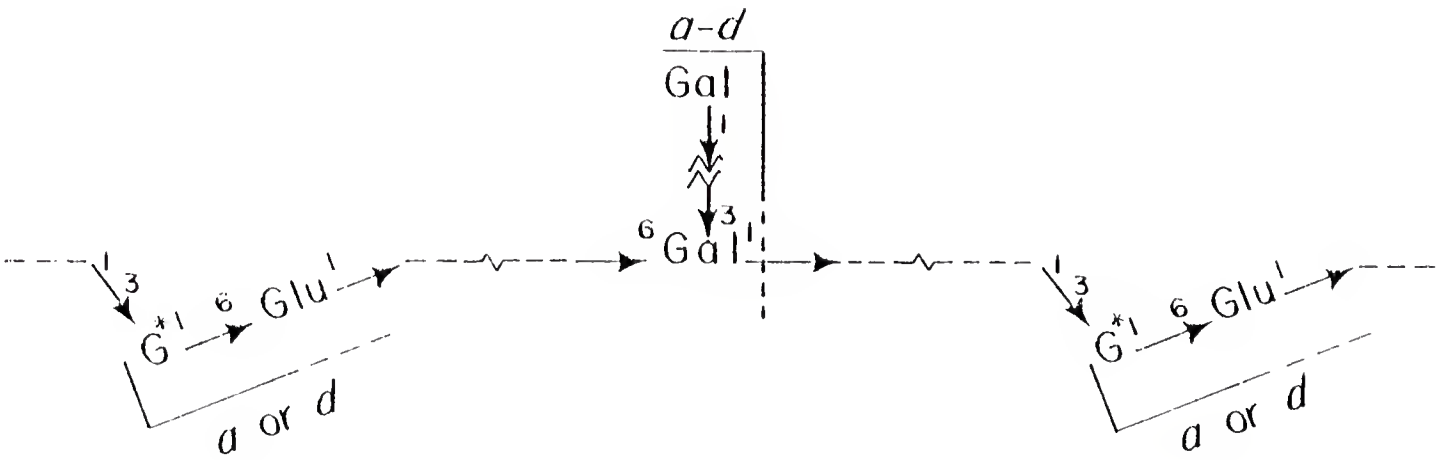
With respect to the specificity of the *d* site on the B13 antigen, the results in Table 6 suggest that the major determinant may be glucopyranoside that is linked 1→6 to another glucose or galactose residue, possibly by means of a β-linkage as indicated by the excellent inhibition obtained using gentiobiose (50%) and β-methyl glucopyranoside (48%). Linzer and Slade (42) meanwhile purified an antigen from cold, 5% trichloroacetic acid (TCA) extracts of whole cells of B13. This antigenic polysaccharide had about the same molar ratio of galactose: glucose (1.9:1) as the B13 antigen isolated and purified in this study (2.1:1). Inhibition of the precipitation of the Linzer-Slade antigen (42) with anti-*d* serum gave the following results: galactose (31%), glucose (5%), rhamnose (0%), lactose (9%), and melibiose (4%). Again, inhibitors were used on an equal weight (500 µg) basis rather than an equimolar basis, despite the fact that a selection of mono- and disaccharides with different molecular weights were being compared. It was also reported that galactosamine, glucosamine, melibiose, cellobiose, stachyose($[Gal-\alpha-1\rightarrow6-]_2$-Glu-α-1→2- fructofuranose), and maltose inhibited less than 5%, again using 500 µg amounts of each. The discrepancy between the published results and those of the present study

cannot readily be explained. There are differences in extraction techniques (formamide on purified cell walls here *vs* 5% TCA on whole cells (42)), purification (affinity chromatography here *vs* gel and ion-exchange chromatography), and in the organisms used to absorb the whole B13 serum to yield anti-*d* immunoglobulins (AHT cells here *vs* HS6 (42)). Again, it is concluded there is much doubt as to the true nature of the *d* determinants, and further studies with additional strains of the serotype are suggested to help resolve the discrepancies.

There is, however, agreement as to the apparent specificity of the *a*-*d* site; namely galactose (probably in the pyranose configuration). This specificity was found using both the AHT and B13 antigens in this study, and in HS6 by Mukasa and Slade (46). (The specificity of the *a*-*d* site of the B13 antigen purified by Linzer and Slade (42) was not determined in that study). Tables 7 and 8 reveal pronounced inhibition by melibiose (72% with B13 and 47% with AHT, each at 10 μmoles). These data suggest that the galactose determinant of *a*-*d* reactivity may reside at the non-reducing end of a branch of the main polymeric chain since melibiose contains galactose linked α-1→6 to glucose.

The data provided by methylation analysis of purified polysaccharides provide much useful information concerning the chemical composition and linkage patterns of each antigen, and when considered with the inhibition data, likely structures for each determinant can be postulated. Although methylation studies do not provide information concerning the sequential arrangement of sugar residues, nor data concerning anomeric linkages, it is possible to derive plausible structures. Figure 16 shows a schematic arrangement of sugar residues in the AHT and B13 antigens taking into account the more important features suggested by the

Figure 16. Schematic arrangement of the sugar residues in the AllT and B13 polymers suggested by the methylation and inhibition data. Only the *a*, *d*, and *a-d* haptenic portions are shown.




G* = Galactose for the *a* determinant

or Glucose for the *d* determinant

methylation analyses and inhibition data. All residues present in each polymer are not shown; only the suggested common ($a$-$d$) and specific ($a$ and $d$) haptenic areas are detailed.

Both polymers possess the same branch-point: a galactose residue linked at C-1,3, and 6 (or a galactofuranose residue linked at C-1,2, and 6). Also, both polymers probably contain a terminal galactose moiety that is always linked to this branch-point since they are present in equimolar amounts. Two other common residues exist: almost 2 moles (relative to the branch) of glucose linked at C-1 and C-6 plus 1.4 moles of galactose linked at the same carbon atoms. The sharing of a terminally-linked galactose residue, and the fact that galactose was the best inhibitor of anti-$a$-$d$ activity, makes the terminal galactose linked to the galactose branch-point a likely arrangement for the $a$-$d$ reactive site. There may be a residue or residues intervening between the terminal galactose and the branch-point (perhaps the common non-terminal galactose linked at C-1 and C-6), however, this cannot be determined at present.

Another important feature found in each polymer is the presence of 1→3 linked galactose (AHT), and glucose (B13). D.A.R. Simmons (55, 56) in studying molecular models of *Shigella flexneri* O-specific side chains found that 1→2 and 1→3 linkages on rhamnose residues caused marked buckling of the polymers, which "broke" the primary chains into sterically discrete repeating sequences which correspond to the antigenic sequences determined by serological procedures. It was also noted that these 1→2 or 1→3 linkages were not involved in determining the antigenic specificity of these determinants but only served to set them apart as sterically discrete and accessible sequences. Simmons proposed that these determinants should be called "apodeterminants" because "by conferring a distinctive shape on

the molecule, they determine the sequence and steric accessibility of the more distant structures that compromise the antigenic determinant without themselves forming an integral part of it" (56). He proposed that the determinants themselves should be designated as "non-terminal determinants." The presence of a galactose residue linked at C-1 and C-3 in the AHT polymer and the fact that inhibition studies show that galactose (possibly linked 1→6 to glucose) is the more inhibitory hexose, support the arrangement shown in Fig. 16. Likewise, the B13 polymer possesses a glucose residue linked at C-1 and C-3 and inhibition studies show that glucose is the better inhibitor of anti-*d* serum activity. Whether the *d* determinant involves a glucose-glucose or a glucose-galactose sequence in a 1→6 linkage is not clear, however both 1→6 linked glucose and galactose residues are found in the B13 polmer. In Fig. 16 the glucose-glucose (1→6) linkage is suggested in light of the fact that glucose residues linked at C-1 and C-6 are so common in this polysaccharide.

Finally, each polymer possesses galactofuranose residues at some location within the chain, and these residues may account for the acid lability of these antigens, as was discussed previously.

More precise determinations of the actual sequence of sugars in each polymer will necessitate isolation of oligosaccharides by partial acid or enzymatic degradation, the determination of their structures, and use of these authentic oligosaccharides as potential inhibitors.

Detailed studies of the fine structure of the *S. mutans* antigens will aid in our greater understanding of the serological relationship among the various strains, and may lead to further subdivision of strains on a serological basis. The precise knowledge of these relationships also will aid in epidemiological studies, and ultimately in the selection of appropriate strains for use in future vaccination programs.

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Thomas Allen Brown is married to the former Mary Elizabeth Byrd.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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